REMARKS

New claims 49-73 are submitted herein. Support for these claims can be found throughout the specification, in particular at page 19, line 7 to page 20, line 15. No new matter is added by way of this amendment.

REJECTION UNDER 35 U.S.C. § 102

Claims 1, 3, 24, 31, 32, 34, 38, and 45 are rejected under 35 U.S.C. § 102(a) as allegedly being anticipated by Ezekiel *et al.*, *Proceedings of ASCO 17*: Abstract No. 1522 (April 15, 1998) ["*Ezekiel*"]. Applicants respectfully traverse this rejection.

The present application is entitled to the priority benefit of U.S. Appl. No 60/085,613, filed May 15, 1998. *Ezekiel* published less than one year before this priority date, and as such, only potentially qualifies as prior art against the present invention under 35 U.S.C. § 102(a).

Harlan Waksal, Mansoor Saleh, and Francisco Robert, named inventors on this application, are co-authors of *Ezekiel*. Therefore, *Ezekiel* does not qualify as prior art under 35 U.S.C. § 102(a) against the present invention. Accordingly, withdrawal of this rejection is respectfully requested.

REJECTIONS UNDER 35 U.S.C. § 103

The Examiner issued several rejections under 35 U.S.C. § 103(a): claims 1, 3, 24, 31-39, 41, 43, 45 and 47 over Bos *et al.*, *ASCO Annual Meeting*, Abstract No. 1381 (1996) ["*Bos*"] in view of Saleh *et al.*, *Proceed. Am. Assoc. Cancer Res. 37*: 612, Abstract #4197 (1996) ["*Saleh*"]; claims 1, 3, 24, 31-41, 43-47 over *Bos* in view of *Saleh* and further in view of Goldstein *et al.*, *Clinical Cancer Res. 1*: 1311-1318 (1995) ["*Goldstein*"]; and claims 1, 3, 24, 31-39, 41-43, 45, 47 and 48 over *Bos* in

view of *Saleh* and further in view of Arnold, U.S. Patent No. 5,736,534 ["*Arnold*"].

Even assuming, *arguendo*, that the combined references establish a *prima facie* case of obviousness, the present claims are still non-obvious over these references. Applicants submit, herewith, the following post-priority date references which establish evidence of secondary indicia of non-obviousness: Bianco *et al.*, *Clinical Cancer Res. 6(11)*:4343-4350 (2000) ["*Bianco*"] and Huang & Harari, *Clinical Cancer Res. 6(6)*:2166-2174 (2000) ["*Huang*"]. Both *Bianco* and *Huang* demonstrate profound synergy of a claimed EGFR inhibitor (c225) in combination with radiation for inhibiting human tumors. None of the art cited by Examiner teaches or suggest such a synergy would be achieved against human tumor cells.

Bianco demonstrates synergy of radiation/anti-EGFR treatment in two different types of human cancer cell lines: ovarian cancer (OVCAR-3) and colon cancer (GEO). Claims 46 and 42 are directed to a method of treating ovarian and colon cancer, respectively. In *Bianco*, two human cancer cell lines

> were treated in a sequential schedule with ionizing radiation followed by MAb C225. A ***supra-additive*** growth inhibitory effect was observed at all doses of MAb C225 and ionizing radiation tested.

Bianco, pg. 4345, col. 2 (emphasis added). Thus, *Bianco* demonstrated an unexpected synergy of radiation combined with anti-EGFR treatment.

Huang also demonstrates synergy of radiation/anti-EGFR treatment in human tumors. *Huang* tested this combination using human squamous cell carcinomas derived from the head and neck (as claimed in claim 45), both *in vitro* and an *in vivo* animal model. *Huang* conclude that

> The *in vivo* tumor response after the combined administration of C225 and radiation is dramatic and long-lasting, as demonstrated within the xenograft model system. Such ***profound antitumor activity*** *in vivo* appears to derive from not only proliferative growth inhibition (with associated cell cycle redistribution), but also from inhibition of postradiation damage repair and inhibition of tumor angiogenesis.

Huang, page 2173, col. 1 (emphasis added). Thus, *Huang* also demonstrated the unexpected synergy of the claimed combination in a claimed cancer treatment.

Nothing in the art relied upon by the Examiner teaches or suggests a synergistic effect could be achieved by administering a combination of radiation/anti-EGFR treatment to human patients. Given the unexpected synergy of radiation/anti-EGFR treatment, the present invention is non-obvious over the cited art. Accordingly, withdrawal of this rejection is respectfully requested.

DOUBLE PATENTING

Claims 1, 3, 24 and 31-48 were provisionally rejected on the ground of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 1-10 of copending Application No. 11/206,825. Application No. 11/206,825 has been abandoned. Accordingly, withdrawal of this rejection is respectfully requested.

As it is believed that all of the rejections set forth in the Official Action have been fully met, favorable reconsideration and allowance are earnestly solicited.

If, however, for any reason the Examiner does not believe that such action can be taken at this time, it is respectfully requested that she telephone Applicants' attorney at (908) 654-5000 in order to overcome any additional objections which he might have.

If there are any additional charges in connection with this requested amendment, the Examiner is authorized to charge Deposit Account No. 12-1095 therefor.

Dated: April 9, 2008

Respectfully submitted,

By Andrea Jo Kamage

Andrea Jo Kamage
Registration No.: 43,703
LERNER, DAVID, LITTENBERG,
KRUMHOLZ & MENTLIK, LLP
600 South Avenue West
Westfield, New Jersey 07090
(908) 654-5000
Attorney for Applicants
Under 37 C.F.R. 1.34

864423_1.DOC

Antitumor Activity of Combined Treatment of Human Cancer Cells with Ionizing Radiation and Anti-Epidermal Growth Factor Receptor Monoclonal Antibody C225 plus Type I Protein Kinase A Antisense Oligonucleotide[1]

Cataldo Bianco, Roberto Bianco, Giampaolo Tortora, Vincenzo Damiano, Patrizia Guerrieri, Paolo Montemaggi, John Mendelsohn, Sabino De Placido, A. Raffaele Bianco, and Fortunato Ciardiello[2]

Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli "Federico II," 80131 Naples, Italy [C. B., R. B., G. T., V. D., S. D. P., A. R. B., F. C.]; Divisione di Radioterapia, Azienda Ospedaliera di Cosenza, Cosenza 77100, Italy [C. B., P. G., P. M.]; University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030 [J. M.]

ABSTRACT

Recent studies have suggested that selective inhibition of mitogenic pathways may improve the antitumor activity of ionizing radiation. The epidermal growth factor receptor (EGFR) is overexpressed and is involved in autocrine growth control in the majority of human carcinomas. Protein kinase A type I (PKAI) plays a key role in neoplastic transformation and is overexpressed in cancer cells in which an EGFR autocrine pathway is activated. We used two specific inhibitors of EGFR and PKAI that are under clinical evaluation in cancer patients: C225, an anti-EGFR chimeric human-mouse monoclonal antibody (MAb); and a mixed-backbone antisense oligonucleotide targeting the PKAI RIα subunit (PKAI AS). We tested in human colon cancer (GEO) and ovarian cancer (OVCAR-3) cell lines the antiproliferative activity of MAb C225 and/or PKAI AS in combination with ionizing radiation. *In vivo* antitumor activity was evaluated in nude mice bearing established GEO xenografts. Dose-dependent inhibition of soft agar growth was observed in both cancer cell lines with ionizing radiation, C225, or PKAI AS oligonucleotide. A cooperative antiproliferative effect was obtained when cancer cells were treated with ionizing radiation followed by MAb C225 or PKAI AS oligonucleotide. This effect was observed at all doses tested in both GEO and OVCAR-3 cancer cell lines. A combination of the three treatments at the lowest doses produced an even greater effect than that observed when two modalities were combined. Treatment of mice bearing established human GEO colon cancer xenografts with radiotherapy (RT), MAb C225, or PKAI AS oligonucleotide produced dose-dependent tumor growth inhibition that was reversible upon treatment cessation. A potentiation of the antitumor activity was observed in all mice treated with RT in combination with MAb C225 or PKAI AS oligonucleotide. Long-term GEO tumor growth regression was obtained following treatment with ionizing radiation in combination with MAb C225 plus PKAI AS oligonucleotide, which produced a significant improvement in survival compared with controls ($P < 0.001$), the RT-treated group ($P < 0.001$), or the group treated with MAb C225 plus PKAI AS oligonucleotide ($P < 0.001$). All mice of the RT + MAb C225 + PKAI AS group were alive 26 weeks after tumor cell injection. Furthermore, 50% of mice in this group were alive and tumor-free after 35 weeks. This study provides a rationale for evaluating in cancer patients the combination of ionizing radiation and selective drugs that block EGFR and PKAI pathways.

INTRODUCTION

Treatment with ionizing radiation induces different biochemical effects in cancer cells, with activation of multiple signaling pathways that lead to either programmed cell death or cell proliferation. The latter effect is probably the result of activation of various mitogenic pathways (1, 2). It has recently been demonstrated that ionizing radiation induces the EGFR[3]/*ras*/*raf*/MAPK pathways through the direct activation of the EGFR tyrosine kinase and the release of TGFα, a specific ligand for EGFR (1, 2). This may be clinically relevant because it could represent a mechanism by which cancer cells become able to escape radiation-induced cell death. In this respect, EGF-related growth factors, such as TGFα, have been implicated in human cancer development and progression through autocrine and paracrine pathways (3). TGFα binds to the extracellular domain

Received 5/31/00; revised 8/17/00; accepted 8/22/0.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This study was supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) and the Consiglio Nazionale delle Ricerche (CNR) Target Project on Biotechnologies.
[2] To whom requests for reprints should be addressed, at Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli "Federico II," Via S. Pansini 5, 80131 Naples, Italy. Phone: 39-081-7462061; Fax: 39-081-7462066; E-mail: fortunatociardiello@yahoo.com.

[3] The abbreviations used are: EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; TGFα, transforming growth factor α; MAb, monoclonal antibody; PKAI, protein kinase A type I; PS, phosphorothioate; MBO, mixed-backbone oligonucleotide; RT, radiotherapy.

of EGFR and activates its intracellular tyrosine kinase domain (3). Ligand binding induces dimerization of EGFR and its autophosphorylation on several tyrosine residues in the intracellular domain, creating a series of high-affinity binding sites for various transducing molecules that are involved in transmitting the mitogenic signal through the *ras/raf*/MAPK pathway (3).

Enhanced expression of TGFα and/or EGFR has been detected in the majority of human carcinomas and has been associated with poor prognosis (3). EGFR overexpression has been also found in human cancer cell lines that are resistant to different cytotoxic drugs (4, 5). For these reasons, blocking of the TGFα-EGFR autocrine pathway has been proposed as a therapeutic target (6). Several pharmacological and biological approaches have been developed for blocking EGFR activation and/or function in cancer cells. Anti-EGFR blocking MAbs, recombinant proteins containing TGFα or EGF fused to toxins, and EGFR-selective tyrosine kinase inhibitors have been characterized for their biological and potentially therapeutic properties (7–15). One of these agents, MAb C225, a chimeric human-mouse IgG1 MAb, has recently entered phase II and III clinical evaluation in cancer patients (16–19).

The cAMP-dependent PKA is an intracellular enzyme involved in controlling cell growth and differentiation (20). The PKAI isoform is overexpressed in human cancer and is directly involved in EGFR mitogenic signaling (21). We have shown that PKAI, through interaction of its RIα subunit with Grb2 adapter protein, has structural interaction with the ligand-activated EGFR, cooperating in the propagation to MAPK of the mitogenic signal (22). Different PKAI inhibitors are under clinical development. Down-regulation of PKAI by unmodified or PS-modified antisense oligonucleotides targeting its RIα subunit causes cell growth inhibition in a variety of human cancer cell lines and has antitumor activity in nude mice (23–25). Modified oligonucleotides of a novel class, defined as MBOs, have been synthesized recently and have significantly improved pharmacokinetic and toxicological properties *in vivo* compared with PS oligonucleotides (26, 27). In this respect, an antisense RIα MBO with hybrid DNA/RNA structure containing 2′-*O*-methyl-ribonucleosides at the 5′ and 3′ ends (PKAI AS), has been synthesized (27). This MBO, named GEM231, has completed phase I clinical trials and has shown an improved safety profile and metabolic stability compared with first-generation PS oligonucleotides (28).

In recent years, there has been a growing interest in combining conventional chemotherapeutic agents with biological agents that selectively inhibit key intracellular targets involved in the process of neoplastic transformation. Previous studies have shown that treatment with MAb C225 or PKAI AS oligonucleotide potentiates the antitumor activity of several cytotoxic drugs in human cancer cells (25, 29–31). In this study, we evaluated whether a similar cooperative effect could be obtained when two human cancer cell lines (GEO and OVCAR-3) were treated with MAb C225 and PKAI AS oligonucleotide in combination with RT.

MATERIALS AND METHODS

Materials. MAb C225 is a human-mouse chimeric anti-EGFR IgG1 MAb, whose biochemical and biological characteristic have been described previously (16). MAb C225 was kindly provided by Dr. H. Waksal (ImClone Systems, New York, NY). PKAI AS is a hybrid oligonucleotide, targeted against the N-terminal 8–13 codons of the RIα regulatory subunit of PKA, with the following sequence, *GCGU*GCCTC-CTCAC*UGGC*. This AS oligonucleotide has been termed GEM231 (28). The control is a scramble MBO obtained by mixing all four nucleosides in a mixture containing all possible sequences. The two oligonucleotides contain PS internucleotide linkages (nucleosides flanking each position are in Roman, and 2′-*O*-methyl-ribonucleoside modifications are in italics). The oligonucleotides were synthesized and kindly provided by Dr. S. Agrawal (Hybridon Inc., Milford, MA).

Cell Lines. Human GEO colon cancer and OVCAR-3 ovarian cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD). The *p53* status of the cancer cell lines is the following: wild-type gene, GEO; point-mutated gene, OVCAR-3 (G-to-A in codon 248). The cell lines were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 4 mM glutamine (ICN, Irvine, United Kingdom) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Ionizing Radiation Treatment and Growth in Soft Agar. Exponentially growing GEO and OVCAR-3 cells were irradiated in 100-mm tissue culture dishes (Becton Dickinson, Lincoln Park, NJ) by a 6 MV photon linear accelerator (General Electric). Following irradiation, cells were trypsinized, and 10^4 cells/well were suspended in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over a base layer containing 0.5 ml of 0.8% agar-medium in 24-well cluster dishes (Becton Dickinson) and was treated every day for a total of 3 days with different concentrations of MAb C225 and/or PKAI AS oligonucleotide. After 10–14 days, the cells were stained with nitroblue tetrazolium (Sigma, St. Louis, MO), and colonies larger than 0.05 mm were counted as described previously (31).

GEO Xenografts in Nude Mice. Female BALB/c athymic (nu+/nu+) mice (5–6 weeks of age) were purchased from Charles River Laboratories (Milan, Italy). The research protocol was approved, and mice were maintained in accordance to institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimated to the University of Naples Medical School Animal Facility for 1 week prior to receiving injections of cancer cells. Mice received s.c. injections of 10^7 GEO cells that had been resuspended in 200 μl of Matrigel (Collaborative Biomedical Products, Bedford, MA). After 7 days, when established tumors ~0.2–0.3 cm^3 in volume were detected, 10 mice/group were treated i.p. with PKAI AS oligonucleotide (5 or 10 mg/kg/dose, injected on days 1–5 of each week for 3 weeks), scramble control oligonucleotide (10 mg/kg/dose, injected on days 1–5 of each week for 3 weeks), or MAb C225 (0.25, 0.5, or 1 mg/dose, injected twice weekly on days 1 and 4 for 3 weeks), or received RT treatment (5, 7.5, or 10 Gy/dose daily, administered on days 1–4). In a subsequent series of experiments, groups of 10 mice bearing established GEO tumors ~0.2–0.3 cm^3 in volume received RT (10 Gy/dose on days 1–4) and/or were treated i.p. with PKAI AS oligonucleotide alone (10 mg/kg/dose, injected on days 1–5 of each

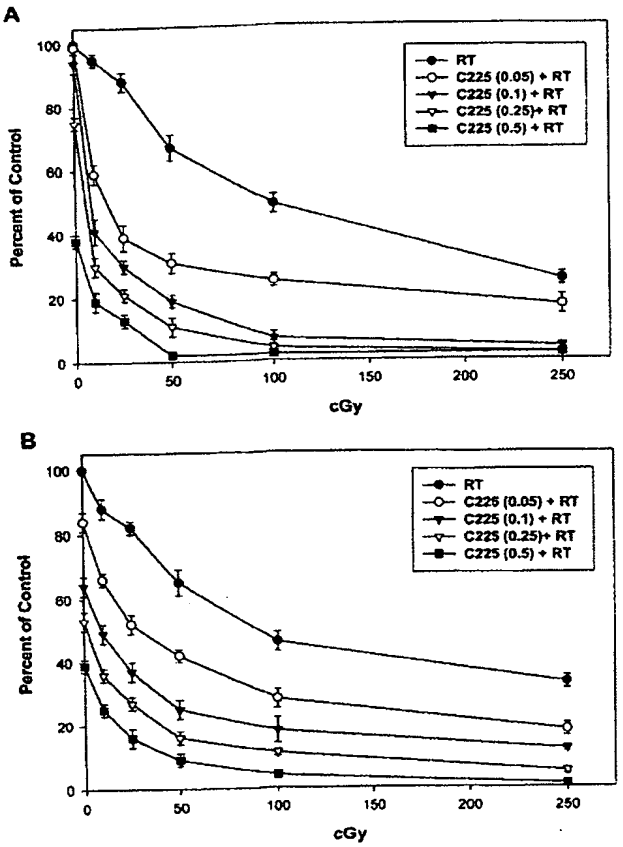

Fig. 1 Dose-dependent growth-inhibitory effects of the combined treatment of ionizing radiation and/or MAb C225 on the soft agar growth of GEO (*A*) and OVCAR-3 (*B*) cells. Cells were treated with different doses of ionizing radiation and/or the indicated concentrations of MAb C225 (μg/ml) as described in "Materials and Methods." Data represent the average of three different experiments, each performed in triplicate; *bars*, SD.

week for 3 weeks), with MAb C225 alone (1 mg/dose, injected twice weekly on days 1 and 4 for 3 weeks), or with both agents. Tumor size was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as reported previously (31).

Statistical Analysis. The Student's *t* test and the Mantel-Cox log-rank test were used to evaluate the statistical significance of the results. All *P* values represent two-sided tests of statistical significance. All analyses were performed with the BMDP New System statistical package, version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA) as reported previously (31).

RESULTS

As shown in Fig. 1, we first evaluated the effects of ionizing radiation and/or MAb C225 treatment on the cloning efficiency of two human epithelial cancer cell lines in soft agar. We selected GEO colon cancer and OVCAR-3 ovarian cancer cell lines because they have functional EGFRs that have ~40,000 (GEO) to 150,000 (OVCAR-3) EGF binding sites/cell and overexpress PKAI (31). GEO cells possess a wild-type *p53* gene, whereas OVCAR-3 cells have a mutated *p53* gene (31). Ionizing radiation treatment caused a dose-dependent inhibition in soft agar growth in both cell lines with an IC_{50} of ~100 cGy. Treatment with the anti-EGFR blocking MAb C225 revealed dose-dependent colony inhibition with an IC_{50} of ~0.3–0.5 μg/ml in both GEO and OVCAR-3 cancer cell lines.

To determine whether combined treatment with ionizing radiation and MAb C225 could enhance the antiproliferative effect of single treatment, the two cancer cell lines were treated in a sequential schedule with ionizing radiation followed by MAb C225. A supra-additive growth inhibitory effect was observed at all doses of MAb C225 and ionizing radiation tested. When lower doses were used in combination, the antiproliferative effect was clearly cooperative in both GEO and OVCAR-3 cells. As an example, in GEO cells, treatment with 25 cGy of ionizing radiation or with 0.25 μg/ml MAb C225 produced ~10 or 22% growth inhibition, respectively, whereas sequential treatment caused 80% inhibition of colony formation in soft agar (Fig. 1*A*). The cooperativity quotient of this treatment, defined as the ratio between the actual growth inhibition obtained with ionizing radiation followed by MAb C225 and the sum of the growth inhibition achieved by each treatment, was ~2.5. We next evaluated whether a similar cooperative antiproliferative effect could be achieved by combining RT with the blockage of PKAI function by a specific PKAI AS oligonucleotide. As illustrated in Fig. 2, in both GEO and OVCAR-3 cells, supra-additive inhibition of cloning efficiency in soft agar was obtained following sequential treatment with ionizing radiation and PKAI AS oligonucleotide. This effect was specific for the combined treatment with the PKAI AS oligonucleotide because treatment with a scramble control oligonucleotide at doses up to 1 μM did not enhance the antiproliferative effect of ionizing radiation in either GEO or OVCAR-3 cancer cells (data not shown).

On the basis of functional and biological interactions between EGFR and PKAI, we previously showed that a concomitant blockade of these two mitogenic pathways may represent a therapeutic strategy (21, 32–34). Therefore, we studied whether any cooperative antiproliferative effect could be obtained when the anti-EGFR blocking antibody MAb C225 and the PKAI AS oligonucleotide were used in combination with RT. Following ionizing radiation, a combination of these two agents caused an even greater inhibitory effect than that observed when a single agent was combined with RT in both GEO and OVCAR-3 cells (Fig. 3). In fact, at single-treatment doses that produced only 5–10% inhibition of colony formation in soft agar, the combination of the three treatments caused 75–85% inhibition of soft agar growth of GEO and OVCAR-3 cells.

We next evaluated whether the cooperative growth inhibitory effect of MAb C225 and PKAI AS following ionizing radiation treatment could also be obtained *in vivo*. GEO cells were injected s.c. into the dorsal flanks of nude mice. When established GEO tumors of ~0.2–0.3 cm^3 were detectable, mice were given RT or were treated i.p. with PKAI AS oligonucleotide or with the anti-EGFR MAb C225. Fig. 4 shows that each treatment significantly inhibited GEO tumor growth *in vivo* in a dose-dependent fashion. However, this effect was reversible

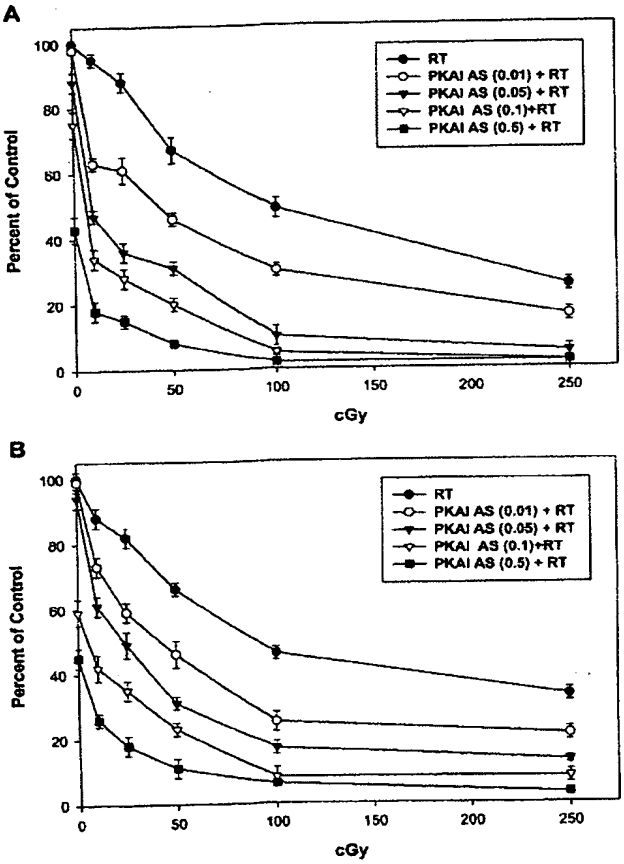

Fig. 2 Dose-dependent growth-inhibitory effects of the combined treatment of ionizing radiation and/or PKAI AS oligonucleotide on the soft agar growth of GEO (*A*) and OVCAR-3 (*B*) cells. Cells were treated with different doses of ionizing radiation and/or the indicated concentrations of PKAI AS oligonucleotide (μM) as described in "Materials and Methods." Data represent the average of three different experiments, each performed in triplicate; *bars*, SD.

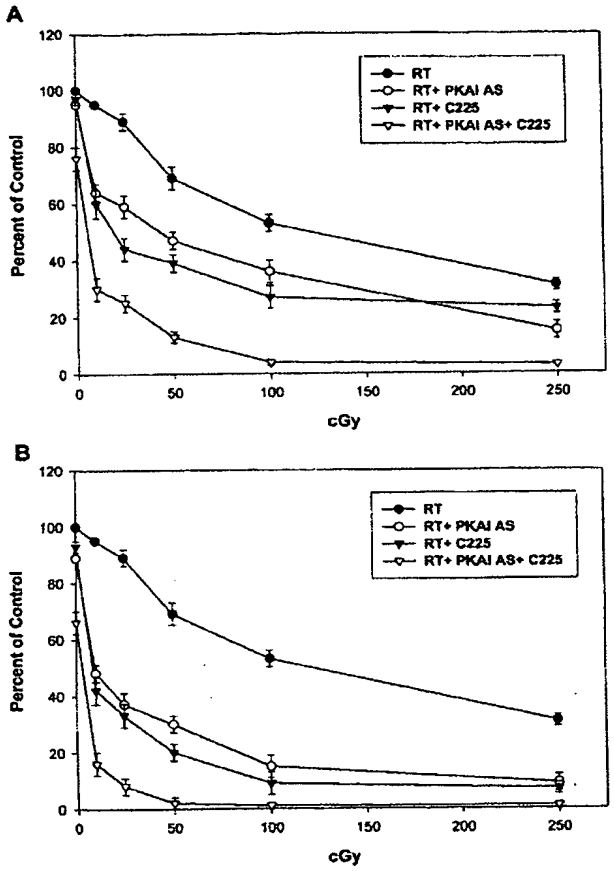

Fig. 3 Dose-dependent growth-inhibitory effects of the combined treatment of ionizing radiation plus MAb C225 and PKAI AS on the soft agar growth of GEO (*A*) and OVCAR-3 (*B*) cells. Cells were treated with different doses of ionizing radiation and/or MAb C225 (0.25 μg/ml) and PKAI AS oligonucleotide (0.1 μM) as described in "Materials and Methods." Data represent the average of three different experiments, each performed in triplicate; *bars*, SD.

because shortly after the end of the treatment with RT, MAb C225, or PKAI AS oligonucleotide, GEO tumors resumed a growth rate similar to that of untreated controls (data not shown). We next evaluated the effects of RT in combination with MAb C225 or with PKAI AS oligonucleotide on mice bearing GEO xenografts. As illustrated in Fig. 5, treatment with MAb C225 (1 mg/dose twice weekly for 3 weeks) after ionizing radiation (10 Gy/dose daily, days 1–4) suppressed tumor growth in all mice. In mice that received MAb C225 plus RT, GEO tumors grew very slowly for ~50–60 days following the end of treatment; tumors then resumed a growth rate similar to controls (Fig. 5*A*). The delayed GEO tumor growth in the MAb C225 + RT-treated group of mice was accompanied by a prolonged life span that was significantly different from controls ($P < 0.001$), the MAb C225-treated group ($P < 0.001$), or the RT-treated group ($P < 0.001$). Furthermore, 20% of mice in this group were still alive without any evidence of tumor 35 weeks after the GEO cancer cells were injected (Fig. 5*B*). A similar but less pronounced potentiation of the antitumor activity of RT was observed when mice were treated with PKAI AS oligonucleotide (10 mg/kg/dose, injected on days 1–5 of each week for 3 weeks; Fig. 6).

We also evaluated the antitumor activity of the triple combination of ionizing radiation treatment and EGFR plus PKAI blockade. Treatment with MAb C225 + PKAI AS oligonucleotide after ionizing radiation produced complete tumor regression in all mice; this regression was maintained for >100 days after the end of treatment (Fig. 7*A*). This effect was reflected in both a significant increase in survival and in a high proportion of cures in the mice receiving the triple-combination treatment. As shown in Fig. 7*B*, GEO tumors reached a size not compatible with normal life in all untreated mice within 6 weeks. A small increase in mouse survival was observed in the group treated with RT alone ($P < 0.05$). Mice treated with MAb C225 + PKAI AS oligonucleotide survived longer than those in the control

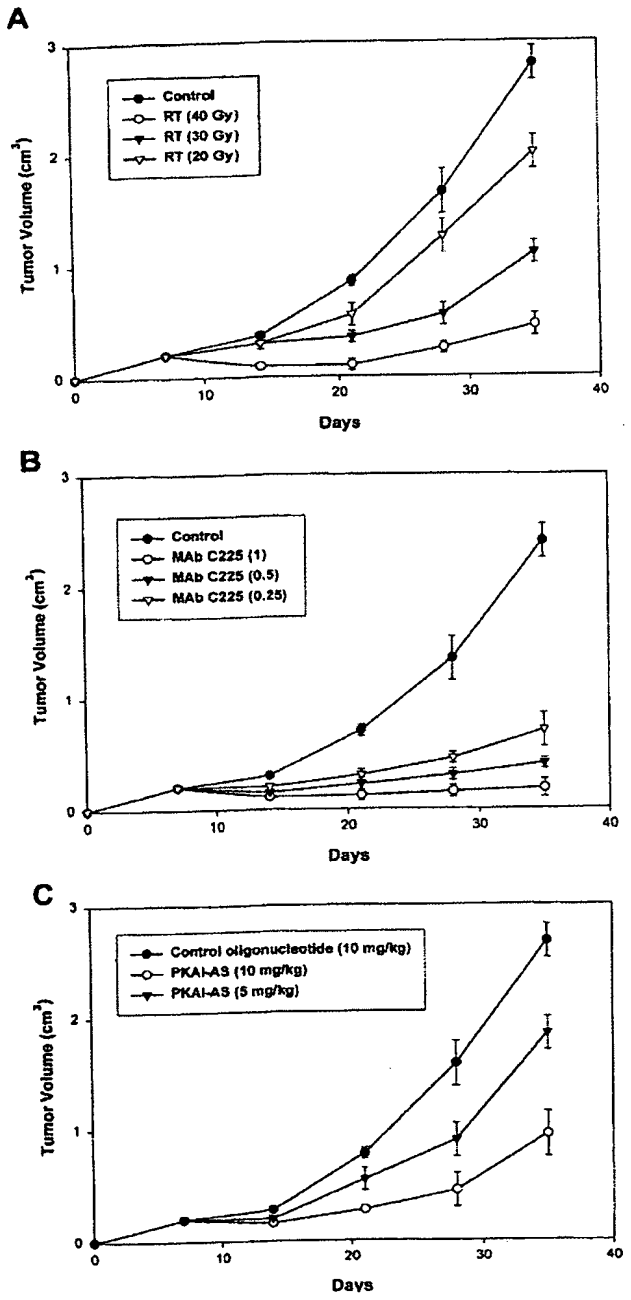

Fig. 4 Antitumor activity of ionizing radiation, MAb C225, or PKAI AS oligonucleotide treatment on established GEO human colon carcinoma xenografts. GEO cells (10^7 suspended in 200 μl of Matrigel) were injected s.c. into the dorsal flanks of mice. After 7 days (average tumor size, 0.2–0.3 cm^3), mice were treated as follows: *A*, ionizing radiation (5, 7.5, or 10 Gy/dose daily, days 1–4 for a total of 20, 30, or 40 Gy); *B*, MAb C225 (0.25, 0.5, or 1 mg/dose i.p., twice weekly on days 1 and 4 for 3 weeks); *C*, PKAI AS (5 or 10 mg/kg/dose i.p., days 1–5 each week for 3 weeks), scramble control oligonucleotide (10 mg/kg/dose i.p., days 1–5 each week for 3 weeks). In each experiment, each group consisted of 10 mice. Data represent the average; *bars*, SD.

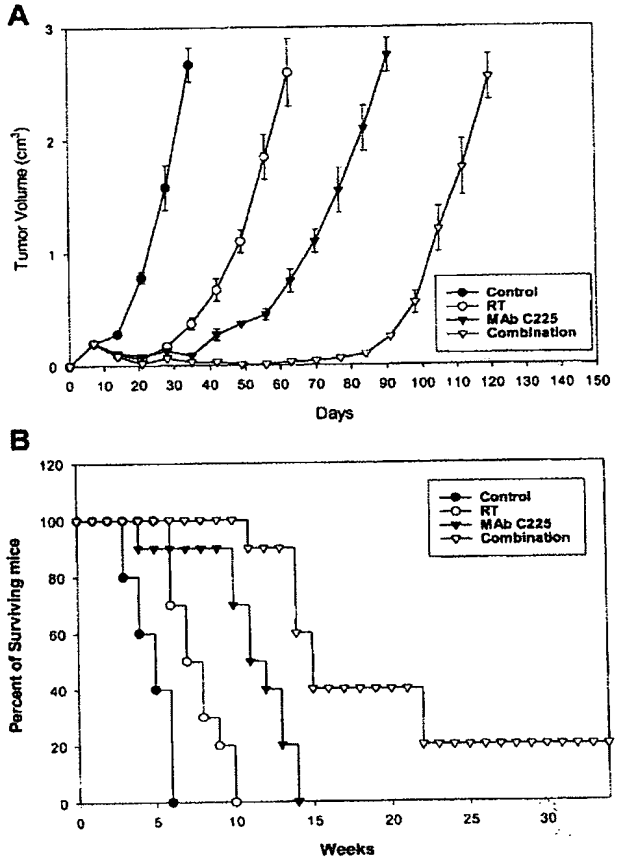

Fig. 5 *A*, antitumor activity of ionizing radiation and MAb C225 on established GEO human colon carcinoma xenografts. GEO cells (10^7 suspended in 200 μl of Matrigel) were injected s.c. into the dorsal flanks of mice. After 7 days (average tumor size, 0.2–0.3 cm^3), mice were treated with ionizing radiation (RT; 10 Gy/dose daily, days 1–4 for a total of 40 Gy) alone, MAb C225 (1 mg/dose i.p., twice weekly on days 1 and 4 for 3 weeks) alone, or with a combination of both. Each group consisted of 10 mice. Data represent the average; *bars*, SD. Student's *t* test was used to compare tumor sizes among different treatment groups at day 28 following GEO cell injection: $P < 0.001$ for MAb C225 *versus* control; $P < 0.001$ for RT *versus* control; $P < 0.001$ for RT followed by MAb C225 *versus* control. *B*, effects of ionizing radiation and/or MAb C225 treatment on the survival of mice bearing GEO tumors. Ten mice per group were monitored for survival. Differences in survival among groups were evaluated using the Mantel-Cox log-rank test. Survival was significantly different between the MAb C225-treated group and the control group ($P < 0.001$), the MAb C225-treated group and the RT-treated group ($P < 0.01$), the RT-treated group and the control group ($P < 0.02$), the RT plus MAb C225-treated group and the control group ($P < 0.001$), the RT plus MAb C225-treated group and the MAb C225-treated group ($P < 0.001$); and the RT plus MAb C225-treated group and the RT-treated group ($P < 0.001$).

group and the RT-treated group ($P < 0.001$). Survival was markedly increased in the group of mice receiving the triple treatment ($P < 0.001$). In fact, all mice of this group were alive 26 weeks after injection of GEO tumor cells. Further-

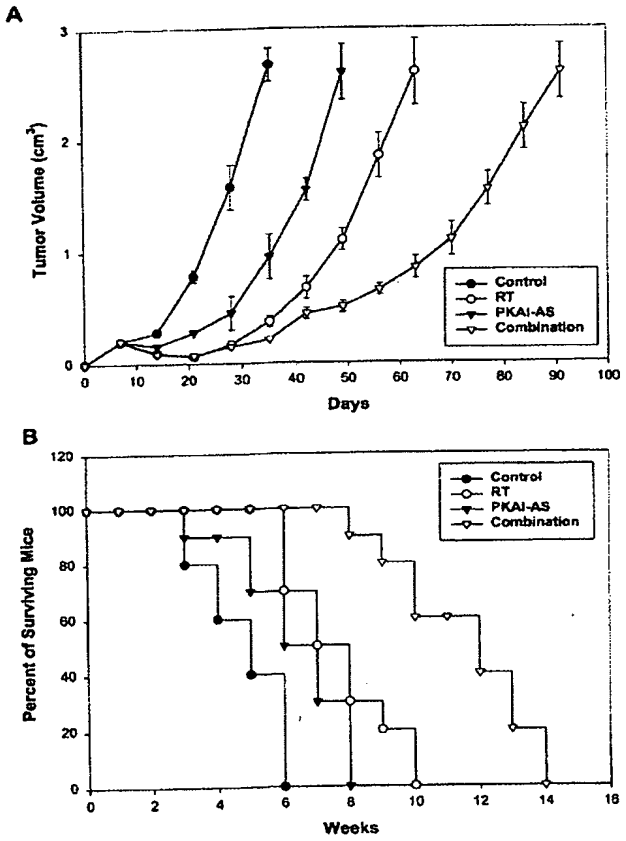

Fig. 6 *A*, antitumor activity of ionizing radiation and PKAI AS oligonucleotide on established GEO human colon carcinoma xenografts. GEO cells (10^7 suspended in 200 μl of Matrigel) were injected s.c. into the dorsal flanks of mice. After 7 days (average tumor size, 0.2–0.3 cm^3), mice were treated with ionizing radiation (RT; 10 Gy/dose daily, days 1–4 for a total of 40 Gy) alone, or PKAI AS (10 mg/kg/dose i.p., days 1–5, each week for 3 weeks) alone, or a combination of both. Each group consisted of 10 mice. Data represent the average; *bars*, SD. Student's *t* test was used to compare tumor sizes among different treatment groups at day 28 following GEO cell injection: $P < 0.001$ for PKAI AS oligonucleotide *versus* control; $P < 0.001$ for RT *versus* control; $P < 0.001$ for RT followed by PKAI AS oligonucleotide *versus* control. *B*, effects of ionizing radiation and/or PKAI AS oligonucleotide treatment on the survival of mice bearing GEO tumors. Ten mice per group were monitored for survival. Differences in survival among groups were evaluated using the Mantel-Cox log-rank test. Survival was significantly different between the RT-treated group and the control group ($P < 0.02$); the RT plus PKAI AS oligonucleotide-treated group and the control group ($P < 0.001$); the RT plus PKAI AS oligonucleotide-treated group and the PKAI AS oligonucleotide-treated group ($P < 0.001$); the RT plus PKAI AS oligonucleotide-treated group and the RT-treated group ($P < 0.001$).

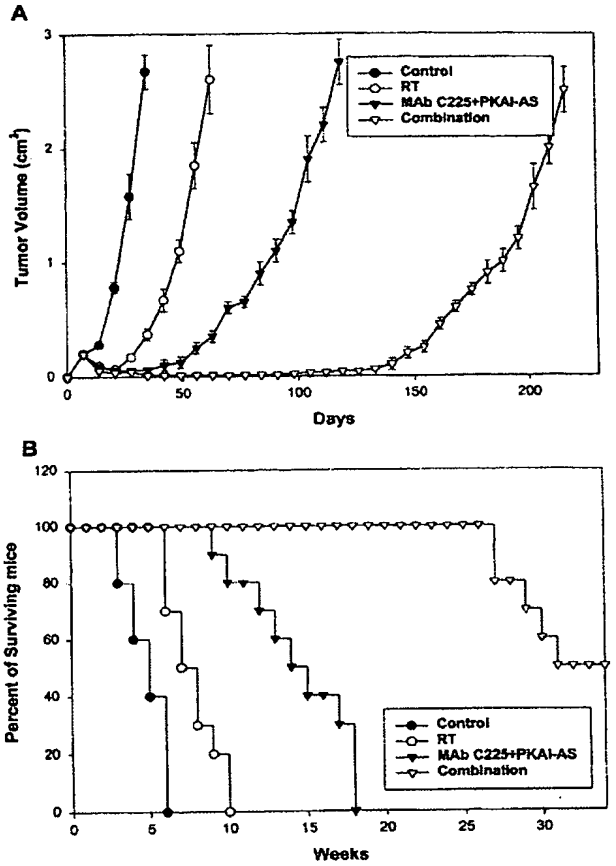

Fig. 7 *A*, antitumor activity of ionizing radiation (RT) and MAb C225 plus PKAI AS oligonucleotide on established GEO human colon carcinoma xenografts. The treatment protocol was the same of the experiments reported in Figs. 5 and 6. Each group consisted of 10 mice. Data represent the average; *bars*, SD. Student's *t* test was used to compare tumor sizes among different treatment groups at day 28 following GEO cell injection: $P < 0.001$ for MAb C225 plus PKAI AS oligonucleotide *versus* control; $P < 0.001$ for RT *versus* control; $P < 0.001$ for RT followed by MAb C225 plus PKAI AS oligonucleotide *versus* control. *B*, effects of ionizing radiation followed by treatment with MAb C225 plus PKAI AS oligonucleotide on the survival of mice bearing GEO tumors. Ten mice per group were monitored for survival. Differences in survival among groups were evaluated using the Mantel-Cox log-rank test. Survival was significantly different between the MAb C225 plus PKAI AS oligonucleotide group and the control group ($P < 0.001$), the MAb C225 plus PKAI AS oligonucleotide group and the RT-treated group ($P < 0.01$), the RT-treated group and the control group ($P < 0.02$), the RT followed by MAb C225 plus PKAI AS oligonucleotide group and the control group ($P < 0.001$), the RT followed by MAb C225 plus PKAI AS oligonucleotide group and the MAb C225 plus PKAI AS oligonucleotide group ($P < 0.001$), and the RT followed by MAb C225 plus PKAI AS oligonucleotide group and the RT-treated group ($P < 0.001$).

more, no histological evidence of GEO tumors was observed in 50% of the mice in this group 35 weeks after tumor cell injection.

DISCUSSION

The possibility of combining conventional anticancer treatments, such as cytotoxic drugs or RT, with novel drugs that selectively interfere with important pathways controlling cancer cell survival, proliferation, invasion, and metastasis has generated a wide clinical interest. This could be a promising therapeutic approach for several reasons. First, because the cellular targets for these agents and their mechanism(s) of action are

different from those of cytotoxic drugs and ionizing radiation, their combination without potential cross-resistance is conceivable. Second, alterations in the expression and/or the activity of genes that regulate mitogenic signals not only can directly cause perturbation of cell growth, but also may affect the sensitivity of cancer cells to chemotherapy and RT (35). In this respect, EGFR overexpression has generally been found in human cancer cell lines that are resistant to different cytotoxic drugs (3–5). Furthermore, ionizing radiation induces the activation of the EGFR tyrosine kinase and the release of its specific ligand, TGFα (2). For these reasons, it has been postulated that EGFR overexpression and activation could be a survival response to counteract apoptotic signals in cancer cells exposed to ionizing radiation or to cytotoxic drugs (2, 35). In fact, it has been proposed that is possible to enhance the anticancer activity by treatment with maximum tolerated doses of cytotoxic drugs or of RT in combination with selective inhibitors of signal transduction pathways instead of increasing chemotherapy or ionizing radiation doses to supertoxic levels that require complex medical support for the cancer patient, such as hematopoietic cell rescue (35).

In the present study, we have shown that treatment with the anti-EGFR blocking chimeric human-mouse antibody MAb C225 potentiates the cytotoxic effects of ionizing radiation in human colon and ovarian cancer cell lines that express functional EGFR. The growth-inhibitory effect *in vitro* is accompanied by a marked increase in antitumor activity *in vivo*, suggesting that the EGFR blockade is able to overcome cancer cell survival signals induced by ionizing radiation treatment. These data are in agreement with and extend those of recent studies by Huang *et al.* (36), who evaluated the effects of MAb C225 on the radiosensitivity of human head-and-neck squamous carcinoma cell lines *in vitro*, and Milas *et al.* (37), who showed *in vivo* enhancement of tumor radioresponse by MAb C225 treatment in nude mice bearing A431 human epidermoid carcinoma xenografts. Furthermore, our study is the first report of a cooperative antiproliferative effect of blocking of PKAI, a serine-threonine kinase acting downstream to EGFR, in combination with RT. The growth-inhibitory effects of MAb C225 and/or PKAI AS treatment in combination with RT seems to be p53-independent because similar results have been obtained in human cancer cells bearing either a normal wild-type or a mutated *p53* gene.

In this study, we also demonstrated that the combined blocking of EGFR and PKAI function and signaling by treatment with MAb C225 and a PKAI AS oligonucleotide following ionizing radiation results in even more efficient cytotoxic activity. In fact, established GEO tumors were eradicated in 50% of mice receiving a relatively short-term treatment with one course of ionizing radiation followed by MAb C225 plus PKAI AS oligonucleotide for 3 weeks.

The results of this study are of potential clinical interest. In fact, they provide a rationale for the combination of MAb C225 and PKAI AS oligonucleotide in the treatment of human epithelial cancers after RT. MAb C225 is in phases II-III clinical development, both alone and in combination with cytotoxic drugs or with RT. In this respect, a pilot phase I study has suggested high antitumor activity of MAb C225 in combination with RT in stage III-IV head-and-neck cancer patients that is maintained as a complete response in 13 of 15 treated patients, with the response lasting 12–27 months (19, 38). Furthermore, the PKAI AS oligonucleotide that we used in the present study has completed phase I evaluation in cancer patients and is in phase II trials (28).

ACKNOWLEDGMENTS

We thank Dr. S. Agrawal for the gift of the MBO oligonucleotides. We also acknowledge the excellent technical assistance of G. Borriello.

REFERENCES

1. Schmidt-Ullrich, R. K., Mikkelsen, R. B., Dent, P., Todd, D. G., Valerie, K., Kavanagh, B. D., Contessa, J. N., Rorrer, W. K., and Chen, P. B. Radiation-induced proliferation of the human A431 squamous carcinoma cells is dependent on EGFR tyrosine phosphorylation. Oncogene, *15:* 1191–1197, 1997.

2. Dent, P., Reardon, D. B., Park, J. S., Bowers, G., Logsdon, C., Valerie, K., and Schmidt-Ullrich, R. K. Radiation-induced release of transforming growth factor α activates the epidermal growth factor receptor and mitogen-activated protein kinase pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. Mol. Biol. Cell, *10:* 2493–2506, 1999.

3. Salomon, D. S., Brandt, R., Ciardiello, F., and Normanno, N. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit. Rev. Oncol. Hematol., *19:* 183–232, 1995.

4. Vickers, P. J., Dickson, R. B., Shoemaker, R., and Cowan, K. H. A multidrug resistant MCF-7 human breast cancer cell line which exhibits cross-resistance to antiestrogens and hormone-independent tumor growth in vivo. Mol. Endocrinol., *2:* 886–892, 1988.

5. Wosikowski, K., Schuurhuis, D., Kops, G. J. P. L., Saceda, M., and Bates, S. E. Altered gene expression in drug-resistant human breast cancer cells. Clin. Cancer Res., *2:* 2405–2414, 1997.

6. Fan, Z., and Mendelsohn, J. Therapeutic application of anti-growth factor receptor antibodies. Curr. Opin. Oncol., *10:* 67–73, 1998.

7. Masui, H., Kamrath, H., Apell, G., Houston, L. L., and Mendelsohn, J. Cytotoxicity against human tumor cells mediated by the conjugate of anti-epidermal growth factor receptor monoclonal antibody to recombinant ricin A chain. Cancer Res., *49:* 3482–3488, 1989.

8. Arteaga, C. L., Hurd, S. D., Dugger, T. C., Winnier, A. R., and Robertson, J. B. Epidermal growth factor receptors in human breast carcinoma cells: a potential selective target for transforming growth factor α-*Pseudomonas* exotoxin 40 fusion protein. Cancer Res., *54:* 4703–4709, 1994.

9. Kirk, J., Carmichael, J., Stratford, I. J., and Harris, A. L. Selective toxicity of TGF-α-PE40 to EGFR-positive cell lines: selective protection of low EGFR-expressing cell lines by EGF. Br. J. Cancer, *69:* 988–994, 1994.

10. Lorimer, I. A. J., Wikstrand, C. J., Batra, S. K., Bigner, D. D., and Pastan, I. Immunotoxins that target an oncogenic mutant epidermal growth factor receptor expressed in human tumors. Clin. Cancer Res., *1:* 859–864, 1995.

11. Schmidt, M., and Wels, W. Targeted inhibition of tumor cell growth by a bispecific single-chain toxin containing an antibody domain and TGFα. Br. J. Cancer, *74:* 853–862, 1996.

12. Buchdunger, E., Mett, H., Trinks, U., Regenass, U., Muller, M., Meyer, T., Beilstein, P., Wirz, B., Schneider, P., Traxler, P., and Lydon, N. 4,5-Bis(4-fluoroanilino)phthalimide: a selective inhibitor of the epidermal growth factor receptor signal transduction pathway with potent *in vivo* antitumor activity. Clin. Cancer Res., *1:* 813–821, 1995.

13. Masui, H., Kawamoto, T., Sato, J. D., Wolf, B., Sato, G., and Mendelsohn, J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. Cancer Res., *44:* 1002–1007, 1984.

14. Aboud-Pirak, E., Hurwitz, E., Pirak, M. E., Bellot, F., Schlessinger, J., and Sela, M. Efficacy of antibodies to epidermal growth factor receptor against KB carcinoma *in vitro* and in nude mice. J. Natl. Cancer Inst., *80:* 1605–1611, 1988.

15. Modjtahedi, H., Eccles, S., Box, G., Styles, J., and Dean, C. Immunotherapy of human tumour xenografts overexpressing the EGF receptor with rat antibodies that block growth factor-receptor interaction. Br. J. Cancer, *67:* 254–261, 1993.

16. Goldstein, N. I., Prewett, M., Zuklys, K., Rockwell, P., and Mendelsohn, J. Biological efficacy of a chimeric antibody to the epidermal growth factor receptor in a human tumor xenograft model. Clin. Cancer Res., *1:* 1311–1318, 1995.

17. Falcey, J., Pfister, D., Cohen, R., Cooper, M., Bowden, C., Burtness, B., Mendelsohn, J., and Waksal, H. A study of anti-epidermal growth factor receptor (EGFr) monoclonal antibody C225 and cisplatin in patients (pts) with head and neck or lung carcinomas. Proc. Am. Soc. Clin. Oncol., *16:* 1364, 1997.

18. Anderson, V., Cooper, M., O'Chei, E., Gilly, J., Falcey, J., and Waksal, H. W. Dose dependent pharmacokinetics of a chimerized monoclonal antibody (C225) against EGFR. Proc. Am. Assoc. Cancer Res., *39:* 3561, 1998.

19. Ezekiel, M. P., Robert, F., Meredith, R. F., Spencer, S. A., Newsome, J., Khazaeli, M. B., Peters, G. E., Saleh, M., LoBuglio, A. F., and Waksal, H. W. Phase I study of anti-epidermal growth factor receptor antibody C225 in combination with irradiation in patients with advanced squamous cell carcinoma of the head and neck. Proc. Am. Soc. Clin. Oncol., *17:* 1522, 1998.

20. Cho-Chung, Y. S., and Clair, T. The regulatory subunit of cAMP-dependent protein kinase as a target for chemotherapy of cancer and other cellular dysfunctional-related diseases. Pharmacol. Ther., *60:* 265–288, 1996.

21. Ciardiello, F., and Tortora, G. Interactions between the epidermal growth factor receptor and type I protein kinase A: biological significance and therapeutic implications. Clin. Cancer Res., *4:* 821–828, 1998.

22. Tortora, G., Damiano, V., Bianco, C., Baldassarre, G., Bianco, A. R., Lanfrancone, L., Pelicci, P. G., and Ciardiello, F. The RIα subunit of protein kinase A (PKA) binds to Grb2 and allows PKA interaction with the activated EGF-receptor. Oncogene, *14:* 923–928, 1997.

23. Yokozaki, H., Budillon, A., Tortora, G., Meissner, S., Beaucage, S. L., Miki, K., and Cho-Chung, Y. S. An antisense oligodeoxynucleotide that depletes RIα subunit of cyclic AMP-dependent protein kinase induces growth inhibition in human cancer cells. Cancer Res., *53:* 868–872, 1993.

24. Nesterova, M., and Cho-Chung, Y. S. A single-injection protein kinase A-directed antisense treatment to inhibit tumour growth. Nat. Med., *1:* 528–533, 1995.

25. Tortora, G., Caputo, R., Damiano, V., Bianco, R., Pepe, S., Bianco, A. R., Jiang, Z., Agrawal, S., and Ciardiello, F. Synergistic inhibition of human cancer cell growth by cytotoxic drugs and mixed backbone antisense oligonucleotide targeting protein kinase A. Proc. Natl. Acad. Sci. USA, *94:* 12586–12591, 1997.

26. Akhtar, S., and Agrawal, S. *In vivo* studies with antisense oligonucleotides. Trends Pharmacol. Sci., *18:* 12–18, 1997.

27. Agrawal, S., and Zhao, Q. Antisense therapeutics. Cur. Opin. Chem. Biol., *2:* 519–528, 1998.

28. Chen, H. X., Marshall, J. L., Ness, E., Martin, R., Dvorchik, B., Rizvi, N., Marquis, J., McKinlay, M., Dahut, W., and Hawkins, M. J. A safety and pharmacokinetic study of mixed-backbone oligonucleotide (GEM231) targeting the type I protein kinase A by two-hours infusions in patients with refractory solid tumors. Clin. Cancer Res., *6:* 1259–1268, 2000.

29. Fan, Z., Baselga, J., Masui, H., and Mendelsohn, J. Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus *cis*-diamminedichloroplatinum on well established A431 cell xenografts. Cancer Res., *53:* 4637–4642, 1992.

30. Baselga, J., Norton, L., Masui, H., Pandiella, A., Coplan, K., Miller, W. H., and Mendelsohn, J. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. J. Natl. Cancer Inst., *85:* 1327–1333, 1993.

31. Ciardiello, F., Bianco, R., Damiano, V., De Lorenzo, S., Pepe, S., De Placido, S., Fan, Z., Mendelsohn, J., Bianco, A. R., and Tortora, G. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. Clin. Cancer Res., *5:* 909–916, 1999.

32. Ciardiello, F., Damiano, V., Bianco, C., di Isernia, G., Ruggiero, A., Caraglia, M., Tagliaferri, P., Baselga, J., Mendelsohn, J., Bianco, A. R., and Tortora, G. Cooperative antiproliferative effects of 8-Cl-cAMP and 528 anti-epidermal growth factor receptor monoclonal antibody on human cancer cells. Clin. Cancer Res., *1:* 161–167, 1995.

33. Ciardiello, F., Damiano, V., Bianco, R., Bianco, C., Fontanini, G., De Laurentiis, M., De Placido, S., Mendelsohn, J., Bianco, A. R., and Tortora, G. Antitumor activity of combined blockade of epidermal growth factor receptor and protein kinase A. J. Natl. Cancer Inst., *88:* 1770–1776, 1996.

34. Ciardiello, F., Caputo, R., Bianco, R., Damiano, V., Pomatico, G., Pepe, S., Bianco, A. R., Agrawal, S., Mendelsohn, J., and Tortora, G. Cooperative inhibition of renal cancer growth by anti-EGF receptor antibody and protein kinase A antisense oligonucleotide. J. Natl. Cancer Inst., *90:* 1087–1094, 1998.

35. Mendelsohn, J., and Fan, Z. Epidermal growth factor receptor family and chemosensitization. J. Natl. Cancer Inst., *89:* 341–343, 1997.

36. Huang, S-M., Bock, J. M., and Harari, P. M. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. Cancer Res., *59:* 1935–1940, 1999.

37. Milas, L., Mason, K., Hunter, N., Petersen, S., Yamakawa, M., Ang, K., Mendelsohn, J., and Fan, Z. *In vivo* enhancement of tumor radioresponse by C225 antiepidermal growth factor receptor antibody. Clin. Cancer Res., *6:* 701–708, 2000.

38. Bonner, J., Ezekiel, M., Robert, F., Meredith, R., Spencer, S., and Waksal, H. Continued response following treatment with IMC-C225, an EGFr MoAb, combined with RT in advancer head and neck malignancies. Proc. Am. Soc. Clin. Oncol., *19:* 5F, 2000.

Advances in Brief

Modulation of Radiation Response after Epidermal Growth Factor Receptor Blockade in Squamous Cell Carcinomas: Inhibition of Damage Repair, Cell Cycle Kinetics, and Tumor Angiogenesis[1]

Shyh-Min Huang, and Paul M. Harari[2]

Department of Human Oncology, University of Wisconsin School of Medicine and Comprehensive Cancer Center, Madison, Wisconsin 53792-0600

Abstract

We have recently demonstrated that molecular blockade of the epidermal growth factor receptor with the anti-epidermal growth factor receptor (EGFR) monoclonal antibody C225 enhances the *in vitro* radiosensitivity of human squamous cell carcinomas (SCCs) derived from the head and neck. In the present study, we further investigated the capacity of C225 to modulate the *in vitro* and *in vivo* radiation response of human SCC tumor cells and xenografts, and we examined several potential mechanisms that may contribute to the enhanced radiation response induced by C225. Tumor xenograft studies demonstrated complete regression of both newly established (20 mm^3) and well-established (100 mm^3) SCC tumors over a 55–100 day follow-up period in athymic mice treated with the combination of C225 (i.p. injection) and radiation. Cell cycle analysis via flow cytometry confirmed that combined treatment with C225 and radiation induced an accumulation of cells in the more radiosensitive cell cycle phases (G_1, G_2-M) with concurrent reduction in the proportion of cells in the more radioresistant S phase. Results from sublethal damage repair and potentially lethal damage repair analyses in cultured SCC cells demonstrated a strong inhibitory effect of C225 on postradiation damage repair. Further, exposure of SCC cells to C225 induced a redistribution of DNA-dependent protein kinase from the nucleus to the cytosol, suggesting one potential mechanism whereby C225 may influence the cellular response to radiation. Immunohistochemical analysis of SCC tumor xenografts after systemic administration of C225 demonstrated inhibition of the *in vivo* expression of tumor angiogenesis markers, including vascular endothelial growth factor and Factor VIII. Taken together, the collective data suggest that the profound *in vivo* antitumor activity identified in the xenograft setting when C225 is combined with radiation derives from more than simply the antiproliferative and cell cycle effects of EGFR system inhibition. In addition to antiproliferative growth inhibition, EGFR blockade with C225 appears to influence the capacity of human SCCs to effect DNA repair after exposure to radiation, and to express classic markers of tumor angiogenesis.

Introduction

Locoregional disease recurrence remains the dominant form of treatment failure for patients with advanced SCC[3] of the H&N. For patients treated with primary radiation therapy, methods to enhance locoregional disease control have commonly included trials with altered fractionation and trials in combination with cytotoxic chemotherapy (1–3). More recently, advances in our understanding and application of molecular biology to cancer therapy provide new opportunities to modulate tumor growth characteristics during treatment. One such approach in the treatment of SCC of the H&N involves modulation of radiation response by EGFR blockade using the anti-EGFR mAb C225 (4, 5). Recent studies have identified C225 as a potent antiproliferative agent in SCC of the H&N, capable of inhibiting tumor cell growth kinetics. In addition, preclinical studies have demonstrated the capacity of C225 to enhance *in vitro* radiosensitivity and to promote radiation-induced apoptosis (6).

Although C225 augmentation of antitumor activity for several chemotherapeutic agents in mouse xenograft models has been demonstrated (7–9), *in vivo* characterization of C225/radiation interactions have not been well established. Several preliminary findings regarding the capacity of C225 to inhibit cellular proliferation (10), to inhibit DNA damage repair (11), and to inhibit tumor angiogenesis (12) suggest mechanisms whereby EGFR blockade might enhance antitumor responses. The experimental studies presented herein were conducted to examine the *in vivo* response of SCC xenografts in athymic mice to dual treatment with radiation and C225, and to explore various mechanisms of C225-mediated enhancement of radiosensitivity.

Received 12/31/99; revised 2/24/00; accepted 2/28/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Grant CA 66786 (to P. M. H.) from the National Cancer Institute. P. M. H is the recipient of a laboratory research award from ImClone Systems Inc., and he holds stock options in the company.

[2] To whom requests for reprints should be addressed, at Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, 600 Highland Avenue, Madison, WI 53792-0600. Phone: (608) 263-8500; Fax: (608) 263-9167; E-mail: harari@mail.humonc.wisc.edu.

[3] The abbreviations used are: SCC, squamous cell carcinoma; bFGF, basic fibroblast growth factor; DNA-PK, DNA-dependent protein kinase; EGFR, epidermal growth factor receptor; FVIII, factor VIII-related antigen; H&N, head and neck; mAb, monoclonal antibody; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; PLDR, potentially lethal damage repair; SLDR, sublethal damage repair; VEGF, vascular endothelial growth factor; PMSF, phenylmethylsulfonyl fluoride.

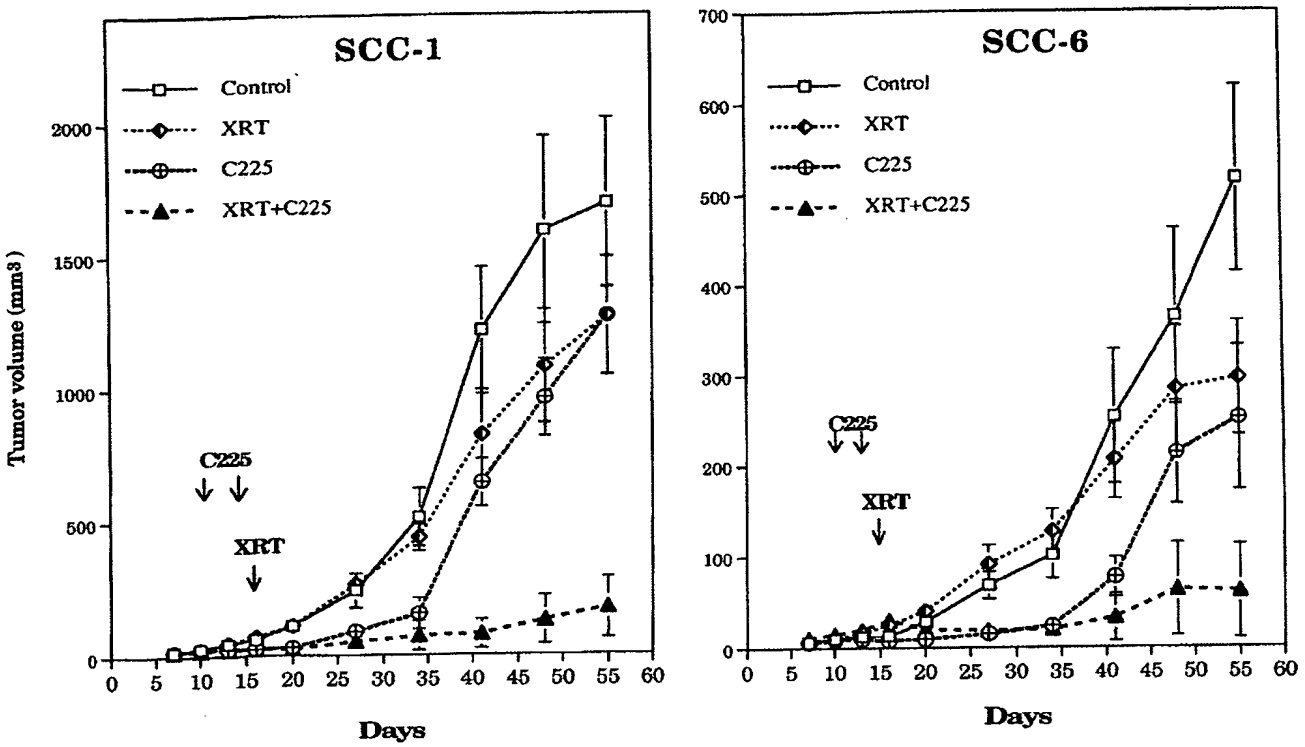

Fig. 1 Antitumor activity of C225 in combination with radiation in newly established SCC xenografts. SCC-1 (10^6) cells (*left panel*) or SCC-6 (5×10^5) cells (*right panel*) were injected s.c. into the dorsal flank of athymic mice as described in "Materials and Methods." Injection of C225 (0.1 mg) was accomplished i.p. on days 10 and 13. Radiation (*XRT*) was delivered with 12 Gy on day 15. *Arrows*, specific days of C225 or XRT administration. Values represent mean tumor size (mm^3) ± SE (n = 6/group).

Materials and Methods

Chemicals and C225. Cell culture media were obtained from Life Technologies, Inc. (Gaithersburg, MD). PI was obtained from Molecular Probes (Eugene, OR). Primary antibody against VEGF, DNA-PK, and PCNA were obtained from Neo Markers (Fremont, CA). Antibody against FVIII (von Willebrand factor) was obtained from DAKO (Glostrup, Denmark). Anti-α-tubulin antibody was obtained from Oncogene Research Products (Cambridge, MA). The enhanced chemiluminescence detection system was purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma (St. Louis, MO). C225 was generously provided by ImClone Systems incorporated (New York, NY).

Cell Lines and Cell Culture. Human SCC cell lines were established from biopsies of H&N cancer patients. The SCC-13Y cell line was derived from the facial epidermis and was provided by Dr. B. Lynn Allen-Hoffman (University of Wisconsin). The SCC-1 cell line (floor of mouth) and SCC-6 cell line (tongue) were provided by Dr. Thomas E. Carey (University of Michigan). The relative expression of EGFR on the cell surface of our SCC cell lines was evaluated via immunofluorescent staining with C225 and subsequent flow cytometry analysis. These SCC cell lines expressed EGFR at essentially comparable levels to those expressed in A431 cells, which are well known to overexpress the EGFR with several million receptors per cell. SCC cells were cultured routinely in DMEM supplemented with 10% Fetal Clone-II serum (Hyclone, Logan, UT), 1 μg/ml hydrocortisone, 1% penicillin, and streptomycin.

Assay of Tumor Growth in Athymic Nude Mice. Athymic Sprague Dawley nude mice (3–4-week-old females) were obtained from Harlan Bioproducts for Science (Indianapolis, IN) and maintained in a laminar air-flow cabinet under aseptic conditions. The care and treatment of experimental animals were in accordance with institutional guidelines. Human SCC cells (~1×10^6) were injected s.c. into the right (SCC-6) and left (SCC-1) flank area of the mice at day 0. Tumor volume was determined by direct measurement with calipers and calculated by the formula: $\pi/6 \times$ (large diameter) $\times$ (small diameter)^2. Animal experiments included four treatment groups: control, radiation alone, C225 alone, and radiation in combination with C225. Control animals received injections of PBS. Radiation treatment was delivered via a precision electron beam from a Varian linear accelerator using custom-designed mouse jigs. These jigs immobilized the animals and specifically exposed the dorsal flank (harboring tumor xenografts) for irradiation without exposing non-tumor-bearing normal tissues. C225 was administered by i.p. injection at the specified doses and intervals.

Immunohistochemical Determination of PCNA, VEGF, and FVIII. The expression of proliferative and angiogenic factors were detected in histological sections of SCC xenografts.

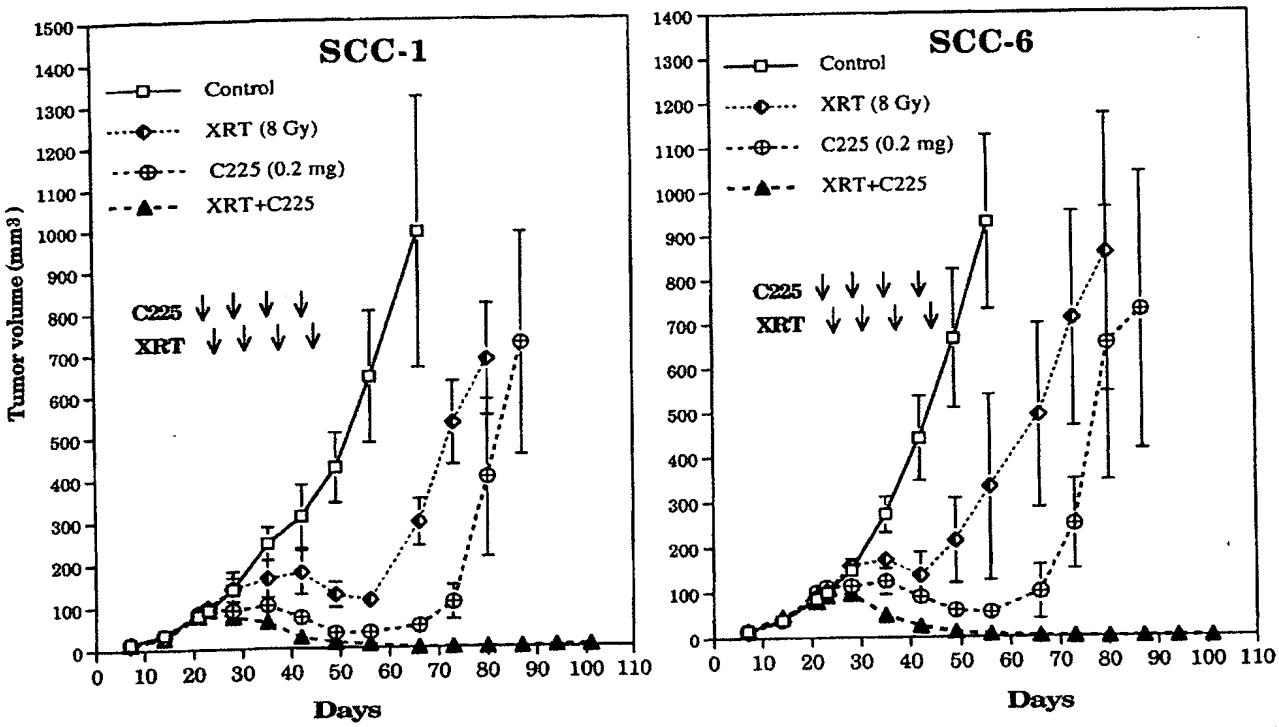

Fig. 2 Antitumor activity of C225 in combination with radiation in well-established SCC xenografts. SCC-1 (10^6) cells (*left panel*) or SCC-6 (10^6) cells (*right panel*) were injected s.c. into the flank of athymic mice as described in "Materials and Methods." After 23 days (tumor mean size of 100 mm^3), treatment was initiated by injecting 0.2 mg of C225 i.p. once a week for a total of four injections. The radiation (*XRT*)-treated group was exposed to a single 8-Gy fraction 24 h after each injection of C225. *Arrows*, specific days of C225 or XRT administration. Values represent mean tumor size (mm^3) ± SE (n = 8/group).

Briefly, excised tumor specimens were fixed in 10% neutral-buffered formalin. After embedding in paraffin, 5-μm sections were cut, and tissue sections were mounted. Sections were dried, deparaffinized, and rehydrated. After quenching endogenous peroxidase activity and blocking nonspecific binding sites, slides were incubated at 4°C overnight with 1:100 dilution of primary antibody directed against PCNA, VEGF, or FVIII followed by a 30-min incubation of biotinylated goat antimouse secondary antibody. Slides were then incubated with streptavidin peroxidase and visualized using the DAB chromogen (Lab Vision Corp., Fremont, CA).

Cell Cycle Analysis. Cell cycle phase distribution after radiation and/or C225 treatment was analyzed by flow cytometry using PI staining. Briefly, control or treated cells were harvested by trypsinization, washed with PBS, then fixed in 95% ethanol and stored at 4°C for up to 7 days before DNA analysis. After the removal of ethanol by centrifugation, cells were incubated with phosphate-citric acid buffer [0.2 M Na_2HPO_4 and 4 mM citric acid (pH 7.8)] at room temperature for 45 min. Cells were then stained with a solution containing 33 μg/ml PI, 0.13 mg/ml RNase A, 10 mM EDTA, and 0.5% Triton X-100 at 4°C for 24 h. Stained nuclei were analyzed for DNA-PI fluorescence using a Becton Dickinson FACScan flow cytometer. Resulting DNA distributions were analyzed by Modfit (Verity Software House Inc., Topsham, ME) for the proportions of cells in G_0-G_1, S phase, and G_2-M phases of the cell cycle.

Immunoblotting Analysis. After treatment, cells were lysed with Tween 20 lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 0.1% Tween 20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10 μg/ml leupeptin and aprotinin] and sonicated. Equal amounts of protein were analyzed by SDS-PAGE. Thereafter, proteins were transferred to nitrocellulose membranes and analyzed by specific primary antibodies against DNA-PK. Proteins were detected via incubation with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence detection system.

Subcellular Fractionation. To examine the effect of C225 on the subcellular distribution of DNA-PK after radiation, control or C225-treated SCC cells were collected and separated into cytoplasmic and nuclear extracts. Briefly, after centrifugation, cells were resuspended in 150 μl of buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 10 μg/ml leupeptin and aprotinin] and incubated on ice for 10 min. The lysate was spun for 30 s to separate the nuclei and supernatant. For cytosol preparation, the supernatant was further centrifuged at 14,000 rpm for 10 min to remove subcellular debris. For nuclear extraction, the nuclei pellet was resuspended in 100 μl of nuclear extraction buffer [20 mM HEPES (pH 7.9), 0.45 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.5 mM PMSF, and 10 μg/ml leupeptin and aprotinin] and incubated for 30 min. Thereafter,

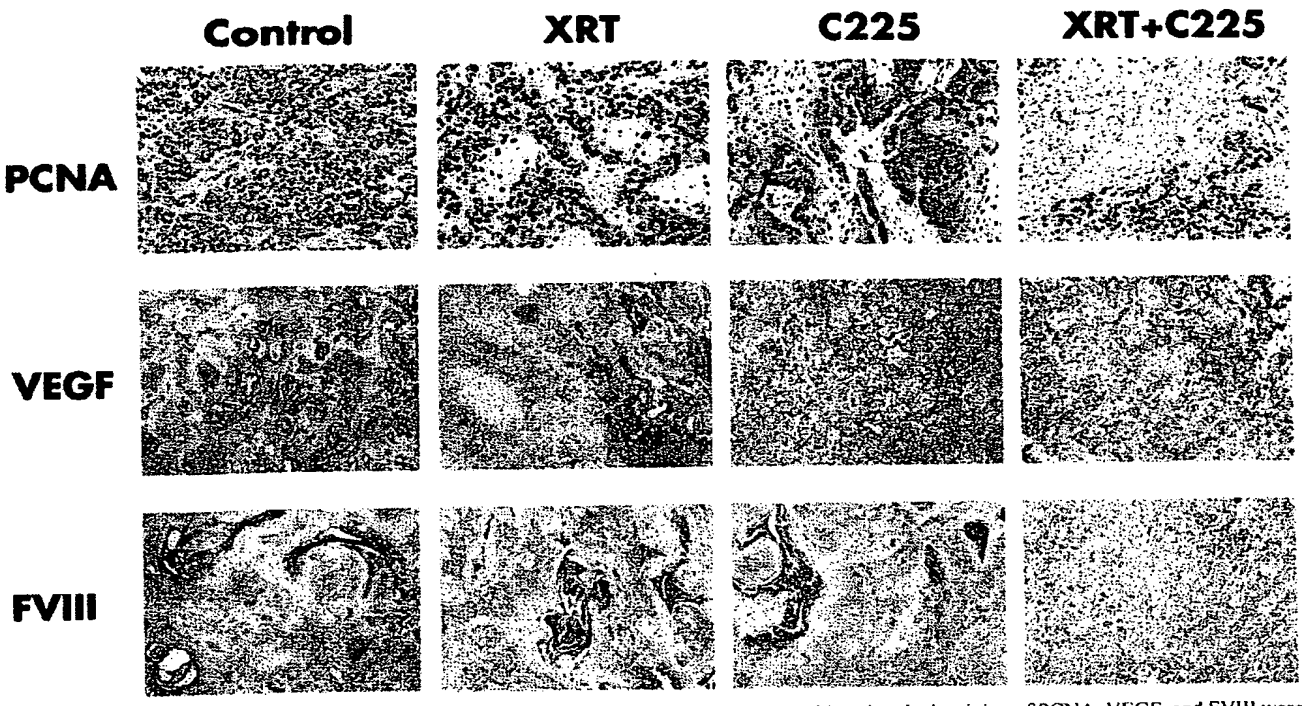

Fig. 3 Effect of C225 on the expression of PCNA, VEGF, and FVIII after radiation. Immunohistochemical staining of PCNA, VEGF, and FVIII were determined using representative human SCC-1 tumor tissue sections taken from mice treated with radiation (*XRT*) alone, *C225* alone, or the combination of radiation and C225 (*XRT+C225*). Positive (red/brown) staining indicates expression of PCNA, VEGF, or FVIII.

the solution was centrifuged at 14,000 rpm for 10 min, and the supernatant (nuclear extracts) was isolated.

Clonogenic Survival Assay. Clonogenic survival was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies. Briefly, after exposure to radiation, cells were trypsinized, counted, and seeded for colony formation in 35-mm dishes at 50–5000 cells/dish. After incubation intervals of 14–21 days, colonies were stained with crystal violet and manually counted. Colonies consisting of ≥50 cells were scored, and 4–10 replicate dishes containing 10–150 colonies/dish were counted for each treatment.

SLDR and PLDR. These studies were designed to examine the influence of C225 on radiation damage repair. SLDR was demonstrated with classic split dose radiation design using a ^{137}Cs irradiator (J. L. Shepherd & Associates, Glendale, CA). Exponentially growing SCC cells received a dose of 3 Gy at time 0 and a second dose of 3 Gy at time points ranging from 2–48 h thereafter. The time 0 point shown in Fig. 4 represents the response to a single dose of 6 Gy. During the time interval between successive 3-Gy fractions, cultures were incubated in the absence (control) or presence of 30 nM C225. After the second radiation exposure, cells were harvested and replated for clonogenic survival analysis as described above. PLDR was demonstrated by single-dose irradiation of confluent cultures, and cell survival was measured by clonogenic assay. The time 0 point shown in the left panel of Fig. 5 represents the survival of cells that were plated immediately after radiation. The remaining data points depict the survival of cells with delayed plating at various time points after their exposure to radiation at time 0.

Statistical Analysis. In all experiments, differences among treatment groups were examined by one-way ANOVA or Student's *t* test using SAS.

Results

C225 Augments *in Vivo* Tumor Response of SCC Xenografts to Radiation. Two human SCC cell lines (SCC-1, SCC-6) were inoculated s.c. into female athymic mice and allowed to grow for 10 days before randomization into four groups. Ten days was the time interval required for xenografts to reach ~20 mm^3 in volume. As shown in Fig. 1, treatment with radiation alone or with C225 alone produced modest inhibition of tumor growth in both SCC-1 and SCC-6 xenografts. In contrast, combined treatment with radiation and C225 produced a marked inhibition in tumor growth over the 55-day observation period in comparison with single modality treatment or control ($P < 0.01$ for all comparisons). In an attempt to examine the *in vivo* interaction of C225 and radiation, low doses of both agents were specifically selected so that their independent effects on tumor growth inhibition would be modest.

We further examined the effect of combining radiation with C225 on the growth response of larger, more well-established SCC xenografts. SCC cells were inoculated as above and allowed to grow until they had achieved a mean volume of 100 mm^3 before C225 treatment. As shown in Fig. 2, C225 was then

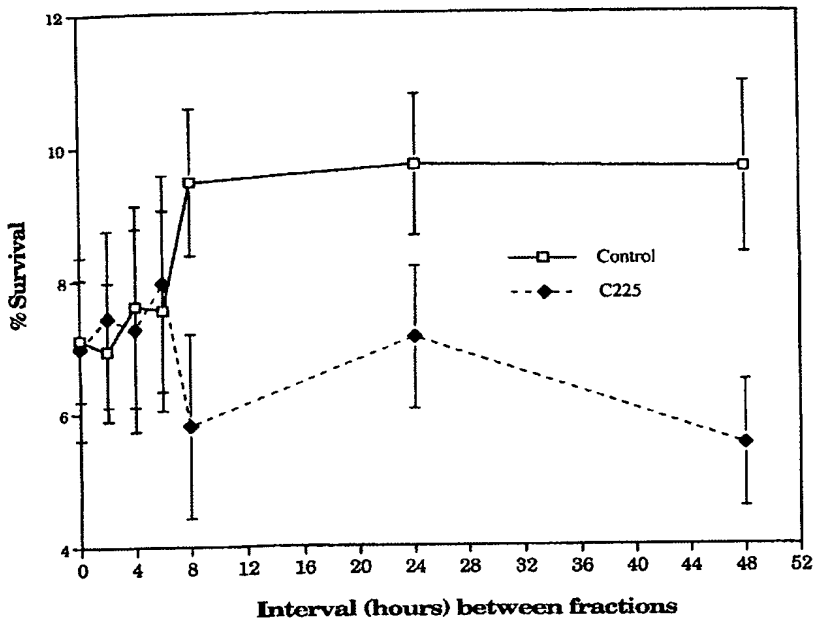

Fig. 4 Inhibition of SLDR by C225. SLDR was examined using a split-dose irradiation technique as described in "Materials and Methods." Exponentially growing SCC-13Y cells were exposed to 3 Gy followed by a second 3-Gy fraction at the indicated time intervals. During the interval between 3-Gy exposures, cultures were incubated in the absence (control) or presence of 30 nM C225. The surviving fraction was then measured by clonogenic assay as described in "Materials and Methods." *Data points*, means of duplicates with five cultures per replicate per time point; *bars*, SD.

administered via i.p. injection on day 23 at a dose of 0.2 mg once a week for 4 consecutive weeks. Radiation fractions were delivered 24 h after each injection of C225 at a dose of 8 Gy. In mice receiving single modality treatment with either radiation or C225, inhibition of tumor growth was observed during the first few weeks after treatment. Thereafter, the tumor growth profile resumed the same growth rate/slope as that of the controls. In contrast, combined treatment with radiation and C225 resulted in dramatic tumor growth inhibition and, in all cases (n = 8 mice), complete tumor regression for up to 100 days of study.

***In Vivo* Expression of PCNA, VEGF, and FVIII.** The expression of several markers of tumor growth and angiogenesis were examined in SCC-1 tumor xenografts. Immunohistochemical staining with PCNA demonstrated the number of proliferating cells to be greatest in the control group, intermediate in the groups receiving single modality treatment with either radiation or C225, and least in the group receiving dual treatment (Fig. 3). Immunostaining for VEGF was far more pronounced in control and radiation groups when compared with groups receiving C225. Using FVIII antibodies directed against endothelial cells, vessel formation was shown to be significantly reduced in the group receiving combined treatment with C225 and radiation compared with that of the remaining groups (Fig. 3). Taken together, these results suggest that C225 may also inhibit tumor angiogenesis in addition to its cell cycle inhibition of proliferation.

SLDR and PLDR. The capacity of C225 to influence SLDR and PLDR was examined in SCC-13Y cells. Fig. 4 depicts the effect of C225 on SLDR after split-dose radiation exposure. Cell survival in control cells shows a steady increase as the time interval between the two radiation fractions increased, which is indicative of SLDR. However, when cells were exposed to C225 during the interval between radiation fractions, cell survival was significantly less ($P < 0.05$) compared with corresponding controls, which is indicative of SLDR inhibition.

The capacity of C225 to influence PLDR was examined under delayed plating conditions for clonogenic survival. The left panel of Fig. 5 shows the time-dependent response of PLDR in SCC-13Y cells. After a single 9-Gy radiation exposure, delayed plating of control cells between 6–48 h resulted in an increased cell survival compared with that observed with immediate plating. In contrast, treatment with C225 before radiation resulted in a reduced survival ($P < 0.02$) in comparison with controls. We further examined the influence of C225 on PLDR when varying doses of radiation (*e.g.*, 0, 3, 6, and 9 Gy) were applied, as shown in the right panel of Fig. 5. As expected, control cells showed a greater survival when they were plated 24 h after radiation rather than immediately after radiation. Conversely, in cells treated with C225, cell survival was not increased by delayed plating, suggesting that PLDR was inhibited by C225. Taken together, these results suggest that C225 compromises the capacity of SCCs to accomplish effective repair after radiation-induced damage.

Subcellular Distribution of DNA-PK. The DNA repair enzyme DNA-PK is known to reside primarily in the nucleus where it exerts a major role in repairing double-strand DNA breaks (13, 14). To examine the effect of C225 on the subcellular localization of DNA-PK after radiation, SCC cells were treated with C225 for 1 h followed by radiation. As expected, a major fraction of DNA-PK was localized in the nucleus, and a minor fraction was localized in the cytosol after radiation exposure (Fig. 6). However, dual treatment with radiation and C225 resulted in a readily measurable shift in the DNA-PK distribution ratio, with an increase in the cytosolic level of DNA-PK and a concurrent reduction in the nucleus. The reduc-

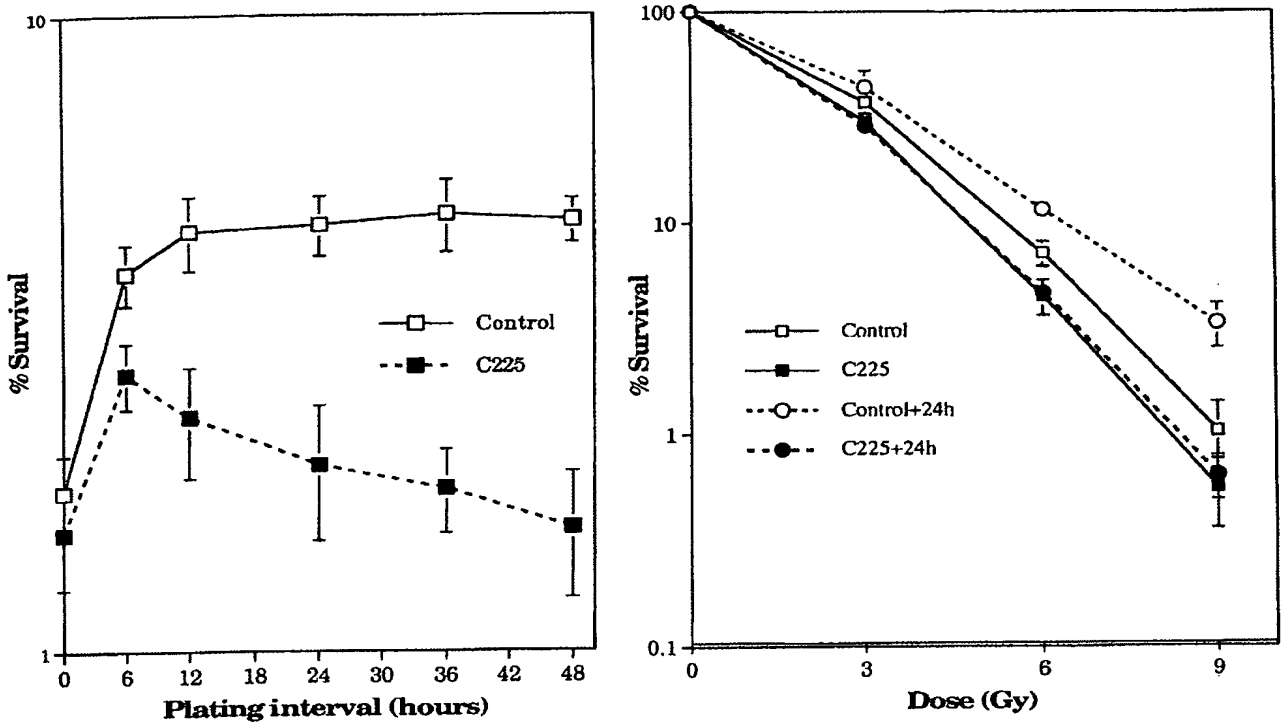

Fig. 5 Inhibition of PLDR by C225. PLDR was examined using single-dose irradiation of confluent cultures as described in "Materials and Methods." Control or C225-treated (30 nM; 72 h) SCC-13Y cells were irradiated with 8 Gy (*left panel*). After irradiation, cells were either immediately subcultured for colony formation (0 h) or were returned to the incubator for delayed plating at specified time intervals. The surviving fraction was then measured by clonogenic assay as described in "Materials and Methods." *Right panel*, radiation dose-response profiles of immediate (□, ■) or 24-h delayed (○, ●) plating for the control group and C225-treated SCC-13Y cells. *Data points*, means of duplicates with five cultures per replicate per point; *bars*, SD.

tion in the level of DNA-PK in the nucleus suggests a potential mechanism whereby the repair of double-strand DNA after radiation may be impaired by C225.

Kinetics and Cell Cycle Progression. Using flow cytometric analysis, the relative distribution of cells among various cell cycle phases were determined in SCC-13Y cells treated with either PBS, radiation, C225, or the combination of radiation and C225. As shown in Fig. 7, G_1 cell cycle arrest was induced in cells exposed to C225 alone, and G_2-M cell cycle arrest was induced in cells receiving radiation alone. Dual treatment with radiation and C225 resulted in the accumulation of cells in both the G_1 and G_2-M phases, with a concurrent reduction of cells within S phase. These results suggest that one potential mechanism whereby C225 may enhance radiosensitivity involves the accumulation of cells in more radiosensitive cell cycle phases, such as G_1 and G_2-M phases.

Discussion

Previous work has demonstrated C225 to be a potent antiproliferative agent capable of inhibiting SCC cell growth in culture (6). In addition, C225 enhances *in vitro* radiosensitivity and promotes radiation-induced apoptosis in SCCs (6). In the present report, we demonstrate that treatment of well-established SCC tumor xenografts with the combination of C225 and radiation can induce complete regression of tumors in athymic mice over a 100-day follow-up period (Fig. 2). The profound antitumor effect observed with combined C225 and radiation (more potent than that observed in cell culture) strongly suggests that mechanisms beyond simple proliferative growth inhibition are operational in the *in vivo* setting. These mechanisms may include (among others) C225-induced inhibition of DNA damage repair, increased radiosensitivity deriving from specific perturbations in cell cycle phase distribution, enhancement of radiation-induced apoptosis, and inhibition of tumor angiogenesis. From the standpoint of cell line variability, recent evidence also identifies a significant inverse relationship between the magnitude of EGFR expression and both radiocurability and radiation-induced apoptosis (15).

In the present studies, the influence of C225 on radiation damage repair was reflected by the capacity of C225 to inhibit PLDR and SLDR. The precise molecular mechanisms that underlie SLDR and PLDR are not clear. Operationally, SLDR is described as that cellular recovery that occurs during the interfraction interval between split-dose radiation exposure, whereas PLDR is described as that cellular recovery that occurs dependent on postradiation conditions after single-dose exposure (16). Both SLDR and PLDR have been shown to be affected by growth factors (17, 18). For example, bFGF has been shown to

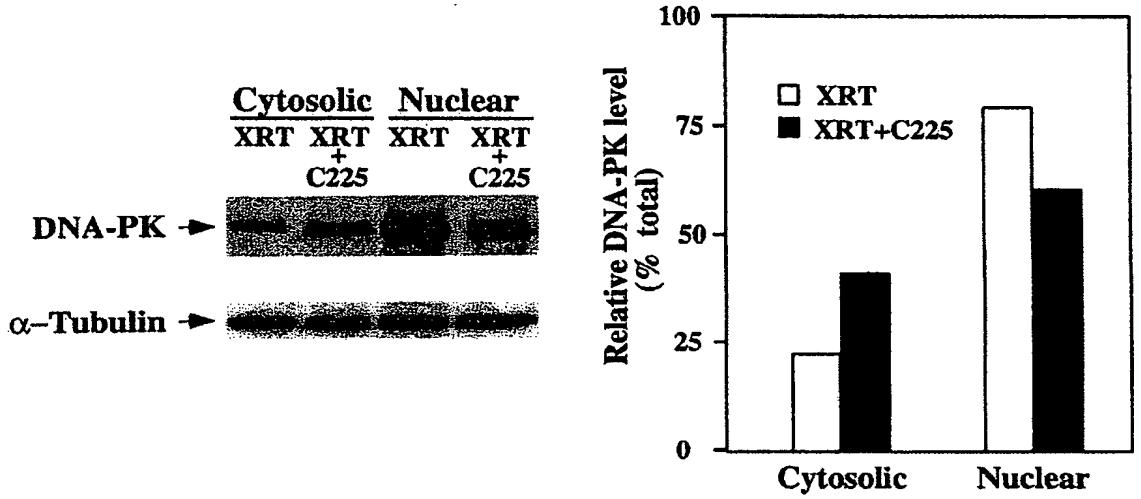

Fig. 6 Effect of C225 on subcellular distribution of DNA-PK. *Left panel*, control or C225-treated (30 nM, 1 h) SCC-13Y cells were irradiated with 2 Gy. After 30-min incubation, cells were then lysed and separated into cytosolic and nuclear extracts as described in "Materials and Methods." After cell lysis, lysates were processed for immunoblotting using antibody directed against DNA-PK as described in "Materials and Methods." α-Tubulin serves as a loading control. *Right panel*, the level of DNA-PK in the samples shown in the *left panel* was densitometrically analyzed using the OFOTO program and quantified by scanner analysis. Results are expressed as the percentage of total level (cytosol + nuclear) in each fraction.

induce PLDR in bovine aortic endothelial cells, and such induction was inhibited by using a neutralizing mAb against bFGF (17). It may be that down-regulation of selected mitogenic signal transduction pathways can inhibit cellular recovery processes after radiation damage, but no precise mechanistic scheme is presently appreciated. A similar postulation has recently been put forth in the HER-2/breast cancer system wherein enhanced radiation response and diminished DNA repair is observed in human MCF-7/HER-2 breast cancer cells after mAb blockade of the HER-2 receptor (19).

Recent evidence has suggested a link between the repair of DNA double-strand breaks and SLDR or PLDR (20). Li *et al.* (21) reported that severe combined immunodeficiency fibroblasts, which were deficient in repairing DNA double-strand breaks, demonstrated a lack of PLDR. Using two isogenic human malignant glioma cell lines, Allalunis-Turner *et al.* (22) demonstrated that cell lines lacking the catalytic domain of DNA-PK did not exhibit SLDR. In our studies, C225 induced a redistribution of DNA-PK with a reduction in the level of DNA-PK in the nucleus of SCC cells exposed to radiation. This finding is consistent with a recent report demonstrating a redistribution of DNA-PK from nucleus to cytosol after exposure to C225 as measured by immunohistochemical analysis (11). Therefore, it is possible that the observed inhibitory effects of C225 on PLDR and SLDR were mediated in part by impairing the function or activity of DNA-PK within the nucleus of SCCs.

The general profile of cell cycle perturbation, which results after exposure to radiation, has been well established (23–25). In general, cells damaged by radiation will arrest in the G_2-M phase presumably to initiate repair of DNA damage before proceeding (26). However, treatment of tumor cells with C225 induces growth arrest primarily in the G_1 phase (10, 27). When tumor cells are simultaneously exposed to modest doses of radiation plus C225 (exposures that individually produced only transient responses in SCC tumor xenografts), profound tumor cell kill is observed, which may reflect a cellular intolerance to concurrent blockade at these two distinct cell cycle checkpoints. Cellular damage induced by radiation signals proliferating cells to pause to initiate repair, and many repair processes require growth factors to proceed effectively. It may be that cells attempting to repair radiation-induced damage, that simultaneously undergo molecular blockade of the EGFR system with agents such as C225, are unable to facilitate effective repair and recovery, thereby contributing to cell death.

The markedly increased antitumor potency of C225 and radiation *in vivo* (over that observed *in vitro*) suggests that factors beyond the confines of the clonogenic cell may influence the *in vivo* response. One possible microenvironmental mechanism involves tumor angiogenesis, which may itself be affected by the functional activity of the EGFR system. Recent findings suggest that the use of an angiogenesis inhibitor (angiostatin) during radiation therapy can significantly enhance response in human tumor xenografts (28). Indeed, several recent associations between the EGFR pathway and tumor angiogenesis have been identified (12, 29, 30). For example, C225 was shown to down-regulate the expression of several angiogenic factors, including VEGF, interleukin 8, and bFGF in A431 and human transitional cell carcinoma of the bladder (12). This down-regulation was postulated to be mediated via reduction of AP-1 activity, which was shared by the promoters of VEGF, bFGF, and interleukin 8. In the present studies, we observed an inhibition of VEGF expression and reduced number of tumor vessels via staining with the endothelial cell marker (FVIII) in xenografts after treatment with C225 and radiation. These findings provide indirect evidence that C225 may also inhibit tumor angiogenesis in addition to the observed effects on radiation-

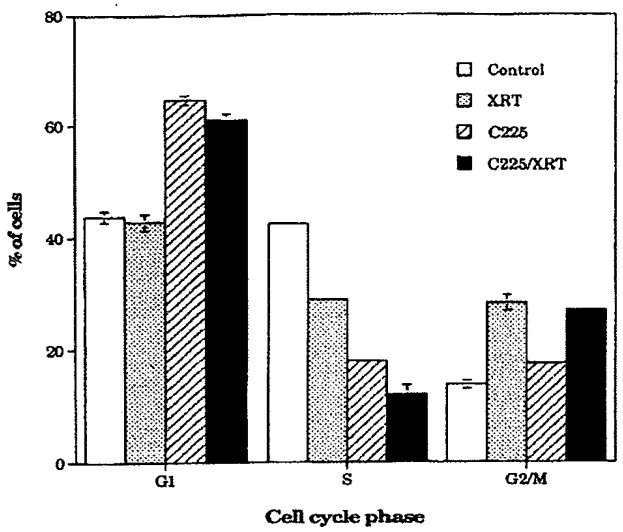

Fig. 7 Effects of C225 on cell cycle progression after radiation. SCC-13Y cells were treated with either PBS (*control*), radiation (*XRT*), *C225*, or the combination of radiation and C225 (*C225/XRT*). Radiation was given at a dose of 3 Gy, and C225 was given at a dose of 30 nM for 24 h. For combined treatment, C225 was given for 24 h followed by radiation. Thereafter, cells were incubated for an additional 24 h before performing flow cytometry analysis as described in "Materials and Methods." DNA histograms were modeled with ModFit analysis software, and phase percentages for G_0-G_1, S phase, and G_2-M are depicted by the bar graph. Data represent mean values of duplicate samples.

induced cytotoxicity. Studies to clarify and augment these findings using complementary *in vivo* assays of angiogenesis are being developed.

In conclusion, epithelial tumors that are rich in their expression of EGFR hold special promise for the receptor blockade approach. SCCs of the H&N are notably robust in their EGFR expression and therefore represent a logical experimental model for EGFR inhibition. In addition, SCCs of the H&N are particularly rapid proliferators, which lends favorably to the antiproliferative impact of EGFR blockade. The studies presented in this report demonstrate that human H&N cancer cells are particularly sensitive to radiation damage when the EGFR signaling pathway in these cells is blocked by C225. Most impressively, the *in vivo* tumor response after the combined administration of C225 and radiation is dramatic and long-lasting, as demonstrated within the xenograft model system.[4] Such profound antitumor activity *in vivo* appears to derive from not only proliferative growth inhibition (with associated cell cycle redistribution), but also from inhibition of postradiation damage repair and inhibition of tumor angiogenesis.

Similar to the recent therapy successes in selected breast cancer and lymphoma patients with mAbs that target specific growth receptor blockade (*e.g.*, herceptin, rituxan), C225 plus radiation therapy in SCC of the H&N represents a promising new molecular cancer therapy approach that has recently commenced formal investigation in Phase III clinical trials.

[4] Similar results demonstrating enchancement of tumor xenograft response with radiation plus C225; communicated to P. M. H. by L. Milas and K. Ang, September 1999.

Acknowledgments

We thank Kathleen Schell and Kristin Elmer for their assistance in the flow cytometry facility at the University of Wisconsin Comprehensive Cancer Center. We also thank ImClone Systems Incorporated for kindly providing us with C225 for experimental studies.

References

1. Ang, K. K., Thames, H. D., and Peters, L. J. Altered fractionation schedules. *In:* C. A. Perez and L. W. Brady (eds.), Principles and Practice of Radiation Oncology, pp. 119–142. Philadelphia: Lippincott-Raven, 1997.

2. Harari, P. M., and Kinsella, T. J. Advances in radiation therapy for head and neck cancer. Curr. Opin. Oncol., *7:* 248–254, 1995.

3. Adelstein, D. J. Recent randomized trials of chemoradiation in the management of locally advanced head and neck cancer. Curr. Opin. Oncol., *10:* 213–218, 1998.

4. Fan, Z., and Mendelsohn, J. Therapeutic application of anti-growth factor receptor antibodies. Curr. Opin. Oncol., *10:* 67–73, 1998.

5. Huang, S. M., and Harari, P. M. Epidermal growth factor receptor inhibition in cancer therapy: biology, rationale and preliminary clinical results. Invest. New Drugs., *17:* 259–269, 1999.

6. Huang, S. M., Bock, J. M., and Harari, P. M. Epidermal growth factor receptor blockade with C225 modulates proliferation, apoptosis, and radiosensitivity in squamous cell carcinomas of the head and neck. Cancer Res., *59:* 1935–1940, 1999.

7. Baselga, J., Norton, L., Masui, H., Pandiella, A., Coplan, K., Miller, W., Jr., and Mendelsohn, J. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. J. Natl. Cancer Inst., *85:* 1327–1333, 1993.

8. Fan, Z., Baselga, J., Masui, H., and Mendelsohn, J. Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus *cis*-diamminedichloroplatinum on well established A431 cell xenografts. Cancer Res., *53:* 4637–4642, 1993.

9. Ciardiello, F., R., B., Damiano, V., De Lorenzo, S., Pepe, S., de Placido, S., Fan, Z., Mendelsohn, J., Bianco, A. R., and Tortora, G. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. Clin. Cancer Res., *5:* 909–916, 1999.

10. Peng, D., Fan, Z., Lu, Y., DeBlasio, T., Scher, H., and Mendelsohn, J. Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates $p27^{KIP1}$ and induces G1 arrest in prostatic cancer cell line DU145. Cancer Res., *56:* 3666–3669, 1996.

11. Bandyopadhyay, D., Mandal, M., Adam, L., Mendelsohn, J., and Kumar, R. Physical interaction between epidermal growth factor receptor and DNA-dependent protein kinase in mammalian cells. J. Biol. Chem., *273:* 1568–1573, 1998.

12. Perrotte, P., Matsumoto, T., Inoue, K., Kuniyasu, H., Eve, B. Y., Hicklin, D. J., Radinsky, R., and Dinney, C. P. N. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. Clin. Cancer Res., *5:* 257–264, 1999.

13. Jackson, S. P., and Jeggo, P. A. DNA double-strand break repair and V(D)J recombination: involvement of DNA-PK. Trends Biochem. Sci., *20:* 412–415, 1995.

14. Finnie, N. J., Gottlieb, T. M., Blunt, T., Jeggo, P. A., and Jackson, S. P. DNA-dependent protein kinase activity is absent in xrs-6 cells: implications for site-specific recombination and DNA double-strand break repair. Proc. Natl. Acad. Sci. USA., *92:* 320–324, 1995.

15. Akimoto, T., Hunter, N. R., Buchmiller, L., Mason, K., Ang, K. K., and Milas, L. Inverse relationship between epidermal growth factor

receptor expression and radiocurability of murine carcinomas. Clin. Cancer Res., *5:* 2884–2890, 1999.

16. Hall, E. J., Marchese, M., Hei, T. K., and Zaider, M. Radiation response characteristics of human cells *in vitro*. Radiat. Res., *114:* 415–424, 1988.

17. Haimovitz-Friedman, A., Vlodavsky, I., Chaudhuri, A., Witte, L., and Fuks, Z. Autocrine effects of fibroblast growth factor in repair of radiation damage in endothelial cells. Cancer Res., *51:* 2552–2558, 1991.

18. Jayanth, V. R., Belfi, C. A., Swick, A. R., and Varnes, M. E. Insulin and insulin-like growth factor-1 (IGF-1) inhibit repair of potentially lethal radiation damage and chromosome aberrations and alter DNA repair kinetics in plateau-phase A549 cells. Radiat. Res., *143:* 165–174, 1995.

19. Pietras, R. J., Poen, J. C., Gallardo, D., Wongvipat, P. N., Lee, H. J., and Slamon, D. J. Monoclonal antibody to HER-2/neu receptor modulates repair of radiation-induced DNA damage and enhances radiosensitivity of human breast cancer cells overexpressing this oncogene. Cancer Res., *59:* 1347–1355, 1999.

20. Frankenberg-Schwager, M., Jha, B., Bar, K., and Frankenberg, D. Molecular mechanism of potentially lethal damage repair. I. Enhanced fidelity of DNA double-strand break rejoining under conditions allowing potentially lethal damage repair. Int. J. Radiat. Biol., *67:* 277–285, 1995.

21. Li, L. Y., Nakajima, H., and Nomura, T. Dose rate effectiveness and potentially lethal damage repair in normal and double-strand break repair deficient murine cells by γ-rays and 5-fluorouracil. Cancer Lett., *123:* 227–232, 1998.

22. Allalunis-Turner, M. J., Zia, P. K., Barron, G. M., Mirzayans, R., and Day, R. S. Radiation-induced DNA damage and repair in cells of a radiosensitive human malignant glioma cell line. Radiat. Res., *144:* 288–293, 1995.

23. Lichter, A. S., and Lawrence, T. S. Recent advances in radiation oncology. N. Eng. J. Med., *332:* 371–379, 1995.

24. Coleman, C. N., and Stevenson, M. A. Biologic basis for radiation oncology. Oncology, *10:* 399–411, 1996.

25. Jung, M., and Dritschilo, A. Signal transduction and cellular responses to ionizing radiation. Semin. Radiat. Oncol., *6:* 268–272, 1996.

26. Iliakis, G. Cell cycle regulation in irradiated and nonirradiated cells. Semin. Oncol., *24:* 602–615, 1997.

27. Wu, X., Rubin, M., Fan, Z., DeBlasio, T., Soos, T., Koff, A., and Mendelsohn, J. Involvement of $p27^{KIP1}$ in G_1 arrest mediated by an anti-epidermal growth factor receptor monoclonal antibody. Oncogene, *12:* 1397–1403, 1996.

28. Mauceri, H. J., Hanna, N. N., Beckett, M. A., Gorski, D. H., Staba, M. J., Stellato, K. A., Bigelow, K., Heimann, R., Gately, S., Dhanabal, M., Soff, G. A., Sukhatme, V. P., Kufe, D. W., and Weichselbaum, R. R. Combined effects of angiostatin and ionizing radiation in antitumour therapy. Nature (Lond.), *394:* 287–291, 1998.

29. Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., and Kerbel, R. S. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am. J. Pathol., *151:* 1523–1530, 1997.

30. Radinsky, R., Risin, S., Fan, D., Dong, Z., Bielenberg, D., Bucana, C. D., and Fidler, I. J. Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. Clin. Cancer Res., *1:* 19–31, 1995.